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(54) Title: POLYKETIDES AND THEIR SYNTHESIS

(57) Abstract

A hybrid type I polyketide synthase gene typically containing a starter module and a plurality of heterologous extender modules is used to synthesise novel polyketides. It is preferably under the control of a type II polypolyketide synthase promoter e.g. act I of S. coelicolor.

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1

POLYKETIDES AND THEIR SYNTHESIS

The present invention relates to novel polyketides and
methods and means for preparing them by recombinant
synthesis. Polyketide biosynthetic genes or portions of
them, which may be derived from different polyketide
biosynthetic gene clusters are manipulated to allow the
production of specific novel hybrid polyketides of
predicted structure. The invention also relates to novel
host-vector systems allowing increased levels of
production of both natural and non-natural polyketides,
both in vivo and in vitro.

Polyketides are a large and structurally diverse class of
natural products that includes many compounds possessing
antibiotic or other pharmacological properties, such as
erythromycin, tetracyclines, rapamycin, avermectin and
FK506. In particular, polyketides are abundantly produced
by Streptomyces and related actinomycete bacteria. They
are synthesised by the repeated stepwise condensation of
acylthioesters in a manner analogous to that of fatty acid
biosynthesis. The greater structural diversity found
among natural polyketides arises from the selection of
(usually) acetate or propionate as "starter" or "extender"
units; and from the differing degree of processing of the
$\Box\beta$-keto group observed after each condensation. Examples
of processing steps include reduction to $\Box\beta$-hydroxyacyl-,
reduction followed by dehydration to 2-enoyl-, and
complete reduction to the saturated acylthioester. The
stereochemical outcome of these processing steps is also
specified for each cycle of chain extension.

The biosynthesis of polyketides is initiated by a group of
chain-forming enzymes known as polyketide synthases. Two
classes of polyketide synthase (PKS) have been described
in actinomycetes. One class, named Type I PKSs,
represented by the PKSs for the macrolides erythromycin,
avermectin and rapamycin (Figure 1), consists of a
different set or "module" of enzymes for each cycle of

2

polyketide chain extension (Figure 2)(Cortes, J. et al.
Nature (1990) 348:176-178; Donadio, S. et al. Science
(1991) 252:675-679; MacNeil, D. J. et al. Gene (1992),
115:119-125; Schwecke, T. et al. Proc. Natl. Acad. Sci.
USA (1995) 92:7839-7843). Note: the term "natural module"
as used herein refers to the set of contiguous domains,
from a β-ketoacyl-ACP synthase ("KS") gene to the next
acyl carrier protein ("ACP") gene, which accomplishes one
cycle of polyketide chain extension. The term
"combinatorial module" is used to refer to any group of
contiguous domains (and domain parts), extending from a
first point in a first natural module, to a second
equivalent point in a second natural module. The first
and second points will generally be in core domains which
are present in all modules, ie both at equivalent points
of respective KS, AT (acyl transferase) or ACP domains.
The length of polyketide formed has been altered, in the
case of erythromycin biosynthesis, by specific relocation
using genetic engineering of the enzymatic domain of the
erythromycin-producing PKS that contains the chain-
releasing thioesterase/cyclase activity (Cortes, J. et al.
Science (1995) 268:1487-1489; Kao, C. M. et al. J. Am.
Chem. Soc. (1995) 117:9105-9106)

In-frame deletion of the DNA encoding part of the
ketoreductase domain in module 5 of the erythromycin-
producing PKS, (also known as 6-deoxyerythronolide B
synthase, DEBS) has been shown to lead to the formation of
erythromycin analogues 5,6-dideoxy-3-α-mycarosyl-5-
oxoerythronolide B, 5,6-dideoxy-5-oxoerythronolide B and
5,6-dideoxy-6,6$\square$-epoxy-5-oxoerythronolide B (Donadio, S.
et al. Science, (1991) 252:675-679). Likewise, alteration
of active site residues in the enoylreductase domain of
module 4 in DEBS, by genetic engineering of the
corresponding PKS-encoding DNA and its introduction into
Saccharopolyspora erythraea, led to the production of
6,7-anhydroerythromycin C (Donadio S. et al. Proc. Natl.

3

Acad. Sci. USA (1993) 90:7119-7123).

International Patent Application number WO 93/13663
describes additional types of genetic manipulation of the
DEBS genes that are capable of producing altered
polyketides. However, many such attempts are reported to
have been unproductive (Hutchinson C. R. and Fujii, I.
Annu. Rev. Microbiol. (1995) 49:201-238, at p.231), and no
further examples of altered polyketides have been
reported. The complete DNA sequence of the genes from
Streptomyces hygroscopicus that encode the modular Type 1
PKS governing the biosynthesis of the macrocyclic
immunosuppressant polyketide rapamycin has been disclosed
(Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci. USA
92:7839-7843)(Figure 3). The DNA sequence is deposited in
the EMBL/Genbank Database under the accession number
X86780.

The second class of PKS, named Type II PKSs, is
represented by the synthases for aromatic compounds. Type
II PKSs contain only a single set of enzymatic activities
for chain extension and these are re-used as appropriate
in successive cycles (Bibb, M. J. et al. EMBO J. (1989)
8:2727-2736; Sherman, D. H. et al. EMBO J. (1989) 8:2717-
2725; Fernandez-Moreno, M. A. et al. J.Biol. Chem. (1992)
267:19278-19290). The "extender" units for the Type II
PKSs are usually acetate units, and the presence of
specific cyclases dictates the preferred pathway for
cyclisation of the completed chain into an aromatic
product (Hutchinson, C. R. and Fujii, I. Annu. Rev.
Microbiol. (1995) 49:201-238). Hybrid polyketides have
been obtained by the introduction of cloned Type II PKS
gene-containing DNA into another strain containing a
different Type II PKS gene cluster, for example by
introduction of DNA derived from the gene cluster for
actinorhodin, a blue-pigmented polyketide from
Streptomyces coelicolor, into an anthraquinone polyketide-
producing strain of Streptomyces galileus (Bartel, P. L.
et al. J. Bacteriol. (1990) 172:4816-4826).

4

International Patent Application Number WO 95/08548
describes the replacement of actinorhodin PKS genes by
heterologous DNA from other Type II PKS clusters, to
obtain hybrid polyketides. The same International Patent
Application WO 95/08548 describes the construction of a
strain of Streptomyces coelicolor which substantially
lacks the native gene cluster for actinorhodin, and the
use in that strain of a plasmid vector pRM5 derived from
the low-copy number plasmid vector SCP2* isolated from
Streptomyces coelicolor (Bibb, M. J. and Hopwood, D. A. J.
Gen. Microbiol. (1981) 126:427) and in which heterologous
PKS-containing DNA may be expressed under the control of
the divergent act I/act III promoter region of the
actinorhodin gene cluster (Fernandez-Moreno, M. A. et al.
J.Biol. Chem. (1992) 267:19278-19290) The plasmid pRM5
also contains DNA from the actinorhodin biosynthetic gene
cluster encoding the gene for a specific activator
protein, Act II-orf4. The Act II-orf4 protein is required
for transcription of the genes placed under the control of
the act I/act III bidirectional promoter and activates
expression during the transition from growth to stationary
phase in the vegetative mycelium (Hallam, S. E. et. al.
Gene (1988) 74:305-320).
Type II PKS clusters in Streptomyces are known to be
activated by pathway-specific activator genes (Narva, K.E.
and Feitelson, J. S. J. Bacteriol. (1990) 172:326-333;
Stutzman-Engwall, K. J. et al. J. Bacteriol (1992)
174:144-154; Fernandez-Moreno, M. et al. Cell (1991)
66:769-780; Takano, E. et al. Mol. Microbiol. (1992)
7:837-845; Takano, E. et al. Mol. Microbiol. (1992)
6:2797-2804) whose gene product is required for
transcription from specific promoters. The gene product
of the activator genes is speculated to act by binding to
specific DNA sequences in promoters of the PKS gene
cluster in which the activator gene is located (Stutzman-
Engwall, K. J. et al. J. Bacteriol (1992) 174:144-154;
Takano, E. et al. Mol. Microbiol. (1992) 7:837-845). The

5

DnrI gene product complements a mutation in the actII-orf4
gene of S. coelicolor, implying that DnrI and ActII-orf4
proteins act on similar targets. A gene (srmR) has been
described (EP 0 524 832 A2) that is located near the Type
I PKS gene cluster for the macrolide polyketide
spiramycin, this gene specifically activates the
production of the macrolide polyketide spiramycin, but no
other examples have been found of such a gene. Also, no
homologues of the ActII-orf4/DnrI/RedD family of
activators have been described that act on Type I PKS
genes.

Although large numbers of therapeutically important
polyketides have been identified, there remains a need to
obtain novel polyketides that have enhanced properties or
possess completely novel bioactivity. The complex
polyketides produced by modular Type I PKSs are
particularly valuable, in that they include compounds with
known utility as antihelminthics, insecticides,
immunosuppressants, antifungal or antibacterial agents.
Because of their structural complexity, such novel
polyketides are not readily obtainable by total chemical
synthesis, or by chemical modifications of known
polyketides.

There is a need to develop reliable and specific ways of
deploying individual modules in practice so that all, or a
large fraction, of hybrid PKS genes that are constructed,
are viable and produce the desired polyketide product.
This is particularly true if it is desired to create large
numbers of individual PKS gene sets using Type I modular
PKS genes in a combinatorial fashion, where it will not be
feasible to analyse all members of the set. Such
libraries of polyketides offer a highly attractive
alternative to the random screening of soil samples for
the discovery of novel polyketides with valuable bioactive
properties.

Similarly, although specific host-vector combinations have
been reported that allow the controlled expression of

6

heterologous genes in certain Streptomyces as for example
using induction by added thiostrepton as described for
Streptomyces lividans 66 and Streptomyces coelicolor
(Takano, E. et al. Gene (1995) 166:133-137) and by
utilising nutritional signals at the onset of
differentiation, as for Streptomyces coelicolor in
International Patent Application number WO 95/08548, there
remains an important need for the development of general
methods of controlling and even enhancing the expression
of a structural gene, or of a set of structural genes,
that governs the biosynthesis of a potentially valuable
secondary metabolite such as one of the complex
polyketides, in an engineered strain of Streptomyces or of
a related filamentous bacterium.

One aspect of the invention arises from our appreciation
that a PKS gene assembly (particularly of type I) encodes
a loading module which is followed by extension modules.
Thus Fig. 2 shows the organisation of the DEBS genes. The
first open reading frame encodes the first multi-enzyme or
cassette (DEBS1) which consists of three modules: the
loading module (ery-load) and two extension modules
(modules 1 and 2). The loading module comprises an acyl
transferase and an acyl carrier protein. This may be
contrasted with Fig. 1 of WO93/13663 (referred to above).
This shows ORF1 to consist of only two modules, the first
of which is in fact both the loading module and the first
extension module.

In one aspect the invention concerns the production of a
hybrid PKS gene assembly comprising a loading module and
at least one, and preferably a plurality, of extension
modules by assembling together a first nucleic acid
portion or portions encoding at least one domain of a
first type I PKS with a second nucleic acid portion or
portions encoding at least one type I PKS domain which is
heterologous to said first PKS. Generally the nucleic
acids are DNA. The first and second portions may each
encode domain(s) of respective different PKS's.

Preferably the hybrid PKS encodes a loading module and
from 1 to 6 extension modules within any give cassette.
More preferably there are at least 2 extension modules.
NB: products resulting from many more than 6 modules can
result from assemblies of synthases (c.f. rapamycin).
The first portion may encode a loading module, while the
second portion encodes one or more extension modules.
Alternatively the first portion(s) may encode all or part
of a loading module, the first two extension modules, and
a chain terminating enzyme (generally a thioesterase),
e.g. of erythromycin PKS, and the second portion(s)
correspond to one or more domains and/or modules of a
different PKS.

It is particularly useful to provide a hybrid PKS gene
assembly in which the loading module is heterologous to
the extension modules and is such as to lead to a
polyketide having an altered starter unit. NB: This is a
concept quite unknown to the prior art since this does not
recognise the existence of loading modules. WO93/13663
refers to altering PKS genes by inactivating a single
function (i.e. a single enzyme) or affecting "an entire
module" by deletion, insertion or replacement thereof.
But in their terms the loading assembly is not a module.
If the loading module is one which accepts many different
carboxylic acid units then the hybrid gene assembly can be
used to produce many different polyketides. For example a
hybrid gene assembly may employ nucleic acid encoding an
avr loading module with ery extender modules. A loading
module may accept unnatural acid units. Alternatively or
additionally we may alter the end of a gene assembly.
Thus the normal chain terminating enzyme of a PKS (usually
thioesterase) may be replaced by an enzyme leading to a
different type of product. Thus use may be made of the
enzyme from the rapamycin system that connects the
polyketide chain to an aminoacid chain. This can be used
to synthesise polypeptide/polyketide combinations, e.g.
for producing $\Box\beta$-lactam derivatives.

8

Of course one may make alterations within a product
polyketide, particularly by replacing an extension module
by one that gives a ketide unit at a different oxidation
state and/or with a different stereochemistry. NB: It has
generally been assumed that the stereochemistry of the
methyl groups in the polyketide chain is determined by the
acyltransferase. But it is in fact a feature of other
domains of the PKS, and thus open to variation only by
replacement of those domains, individually or by module
replacement. Methyl and other substituents can be added
or removed by acyltransferase domain replacement or total
module replacement.

This aspect of the invention is largely concerned with
treating PKS gene modules as building blocks that can be
used to construct enzyme systems, and thus polyketide
products, of desired types. This generally involves the
cutting out and the assembly of modules and multi-module
groupings. It might be assumed that the correct places
for making and breaking intermodular connections would be
in the linking regions between modules, where our
previously-reported experiments using limited proteolysis
have shown those linkers to be on the surface of the
protein (Aparicio, J. F. et al. (1994) J. Biol. Chem.
269:8524-8528; Staunton, J. et al. (1996) Nature
Structural Biol. 3:188-192) . However we have found that
it may be preferable to make cuts and joins actually
within domains (i.e. the enzyme-coding portions), close to
the edges thereof. The DNA is highly conserved here
between all modular PKS's, and this may aid in the
construction of hybrids that can be transcribed. It also
assists in maintaining the spacing of the active sites of
the encoded enzymes, which may be important. For example
in producing a hybrid gene by replacing the ery loading
module by an avr loading module, we removed the ery module
together with a small amount of the following ketosynthase
(KS) domain. The start of the KS domain (well spaced from
the active site) is highly conserved and therefore

9

provides an alternative splicing site to the obvious site
in the linker region between loading module and KS domain.
The excised ery module was then replaced by an avr loading
module.

In fact when substituting a loading module, it may be
desirable to replace not just the loading module domains
(generally acyl transferase (AT) and acyl carrier protein
(ACP)) but also the KS at the start of the following
extension module. Typically the excised loading module
would have provided a propionate starter, and the
replacement is intended to provide one or more different
starters. But propionate may feed in to the KS of the
extension module from a propionate pool in the host cell,
leading to dilution of the desired products. This can be
largely prevented by substituting an extended loading
module including all or most of the KS domain. (The
splice site may be in the end region of the KS gene, or
early in the following AT gene, or in the linker region
between the KS and AT domains.)

When replacing "modules", we are not restricted to
"natural" modules. For example a "combinatorial module"
to be excised and/or replaced and/or inserted may extend
from the corresponding domain of two natural-type modules,
e.g. from the AT of one module to the AT of the next, or
from KS to KS. The splice sites will be in corresponding
conserved marginal regions, or in linker regions between
domains near known sites for limited proteolysis. A
combinatorial module can also be a 'double' or larger
multiple, for adding 2 or more modules at a time.

The invention further provides such gene assemblies,
vectors containing such gene assemblies, and transformant
organisms that can express them. Transformant organisms
may harbour recombinant plasmids, or the plasmids may
integrate. A plasmid with an *int* sequence will integrate
into a specific attachment site (*att*) of a host's
chromosome. Transformant organisms may be capable of
modifying the initial products, e.g. by carrying out all

10

or some of the biosynthetic modifications normal in the
production of erythromycins (as shown in Fig 2B) and/or
other polyketides. Use may be made of mutant organisms
such that some of the normal pathways are blocked, e.g. to
produce products without one or more "natural" hydroxy-
groups or sugar groups. The invention further provides
novel polyketides as producible, directly or indirectly,
by transformant organisms. (This includes polyketides
which have undergone enzymic modification in the organisms
and/or have been isolated and subjected to chemical
modification.)

In a second aspect the invention provides a hybrid gene
assembly comprising structural gene components operably
linked to a promoter which is not naturally linked thereto
and is of a type II PKS, preferably linked to its specific
cognate activator gene. Particularly preferred is the use
of the act I promoter and the Act II- orf4 activator gene
from S.coelicolor, for expression in hosts other than
S.coelicolor (usually other actinomycetes, particularly
other streptomycetes). The structural gene components may
be of a type I PKS gene system.

The invention in its second aspect further provides
vectors containing such gene assemblies, and transformant
organisms that can express them. It is possible to
combine the two aspects of the invention, so that a hybrid
type I gene is expressed under the control of a type II
promoter.

In a further aspect the invention provides novel
polyketides obtainable by means of the previous aspects.
These include the following.

(i)   An erythromycin analogue (being a macrolide compound
with a 14-membered ring) in which C-13 bears a side-chain
other than ethyl, generally an □-branched C_2-C_5 alkyl
group, a C_3-C_8 cycloalkyl or cycloalkenyl group (optionally
substituted e.g. with one or more hydroxy, C_{1-4} alkyl or
alkoxy groups or halogen atoms), or a 3-6 membered
heterocycle containing O or S, saturated or fully or

SUBSTITUTE SHEET (RULE 26)

11

partially unsaturated, optionally substituted (as for cycloalkyl). Preferred candidates for the C-13 substituent R are the groups of carboxylate units R. CO.0- usable as substrates by an avr starter module, or rapamycin starter variants. Preferred substrates include isobutyrate (R=i-Pr) and 2-methylbutyrate (R=1- methylpropyl). Other possibilities include n-butyrate, cyclohexyl carboxylate, cycloheptanyl carboxylate, cyclohexenyl carboxylates, cycloheptenyl carboxylates, and ring-methylated variants of the cyclic carboxylates.

The erythromycin analogue may correspond to the initial product of a PKS (6-deoxyerythronolide) or the product after one or more of the normal biosynthetic steps. As shown in Fig. 2B these comprise: 6-hydroxylation; 3-0- glycosylation; 5-0-glycosylation; 12-hydroxylation; and specific sugar methylation.

Thus the analogues include ones corresponding to 6- deoxyerythronolide B, erythromycin A, and the various intermediates and alternatives shown in Fig. 2B. Additionally or alternatively, there may be chemical modification. For example one or more hydroxy groups may be oxidised (e.g. to produce 3-keto derivatives) or eliminated (e.g. to produce 10-ene derivatives). Some examples of chemical modifications applicable to the present inventions are those that give rise to azithromycin, roxithromycin, clarithromycin and those disclosed in some French patents of Roussel Uclaf:2697523, 2697524 and 2702480.

(ii) erythromycin analogues differing from the corresponding 'natural' compound (Figure 2a) in the oxidation state of one or more of the ketide units (i.e. selection of alternatives from the group: -CO-, -CH(OH)-, =CH-, and -CH$_2$-).

The stereochemistry of any -CH(OH)- is also independently selectable.

(iii) erythromycin analogues differing from the corresponding 'natural' compound in the absence of a

12

'natural' methyl side-chain.   (This is achievable by use
of a variant AT). Normal extension modules use either C_2 or
C_3 units to provide unmethylated and methylated ketide
units. We may provide unmethylated units where methylated
units are natural (and vice versa, in systems where there
are naturally unmethylated units) and also provide larger
units, e.g. C_4 to provide ethyl substituents.

(iv)   erythromycin analogues differing from the
corresponding 'natural' compound in the stereochemistry of
'natural' methyl; and/or ring substituents other than
methyl.

(v)   erythromycin analogues having the features of two or
more of sections (i) to (iv);

(vi)   triketide lactone ("TKL") analogues:
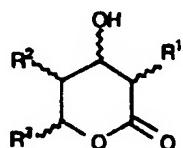
(I)

R_3 is the side-chain derived from the starter unit, and is
subject to the variation described for the C-13 sidechain
described above in (i).

R_1 and R_2 are "naturally" methyl but either or both may be
replaced by hydrogen or ethyl (using extender units
employing butyrate)

The natural stereochemistry is

(II)

13
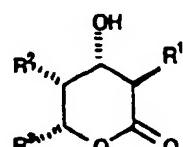
but any one or two or all of R_1, R_2, R_3 and OH may have the opposite stereochemistry. Generally TKL analogues can have variations as described for erythromycins in (i) to (v) above.

(vi) polyketides of types other than erythromyin, e.g. rapamycin or avermectin, having modifications corresponding to those described in sections (i) to (v). For example, we have produced rapamycin variants using as added starter acids:
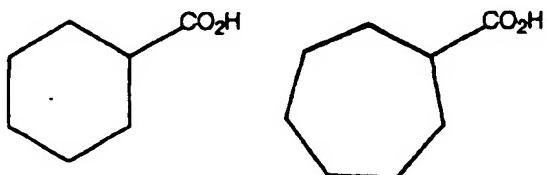
viii) truncated or extended versions of polyketide chains:
a) diketides R^1-CHOH-CHR^2-CO$_2$H
b) triketides R^1-CHOH-CHR^2-CHOH-CHR^3-CO$_2$H
c) tetraketides R^1-CHOH-CHR^2-CHOH-CHR^3-CHOH-CHR^4-CO$_2$H
d) penta-, hexa-, hepta- and larger ketide chains
The chains may have variants as described in (i) to (iv).

ix) ketide/non-ketide fusions.
Rapamycin is a natural example of a polyketide/peptide fusion. Means such as a peptide incorporating enzyme may be employed to create polyketides fused to one or more amino acids.

x) Polyketides (or fusions) cyclised by formation of lactones, hemiketals, ketals, lactams, or lactols.

xi) derivatives of any of the above which have undergone further processing by non-PKS enzymes, eg one or more of hydroxylation, epoxidation, glycosylation, and

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14

methylation.

The present invention provides a method of obtaining novel complex polyketides; and novel methods of increasing production of both new and known polyketides.

Thus in one type of embodiment of the invention, one or more segments of DNA encoding individual modules or domains within a natural Type I PKS (the "donor" PKS) have been used to replace the DNA encoding, respectively, individual modules or domains of another natural Type I PKS (the "acceptor" PKS). The total number of extension modules assembled in the hybrid PKS is not fixed, but the preferred number of such modules in any one multienzyme or cassette ranges between one, creating the smallest possible functional PKS, and six, which equals the largest number of consecutive modules found to date to be housed in a single multienzyme of a natural Type I PKS, namely the rap PKS of Streptomyces hygroscopicus.

In a particularly preferred embodiment for the purposes of defining which hybrid PKS genes will be viable and productive, the acceptor PKS DNA consists of, or comprises of, the loading module, first two extension modules and chain-terminating thioesterase of the ery PKS, or other, preferably natural, type I PKS, housed in a suitable plasmid vector. Either one or more individual domains, or one or more individual modules, are specifically replaced by DNA encoding analogous domains or modules and derived from a different natural Type I PKS (the "donor" PKS).

The altered DNA sequence is introduced into a suitable microorganism and the genetically engineered microorganism is cultured under conditions suitable for polyketide production.

Surprisingly and unexpectedly, these genetically engineered microorganisms when cultured under suitable conditions·have been found to produce non-natural analogues of the polyketide product(s) of the natural acceptor PKS, and where appropriate the products are found to undergo the same processing as the natural polyketide.

15

In this aspect of the invention, the plasmid vector may be any one drawn from a long list of plasmid vectors well known to be useful for cloning in Streptomyces and related Gram positive bacteria. It has been found particularly useful to select a low copy number plasmid vector with a broad host range based on the SCP2* plasmid of Streptomyces coelicolor M110. The construction is described herein of two SCP2*-derived plasmids particularly suitable for this purpose. A precursor plasmid in the construction of one of these two plasmids, lacking a streptomycete origin of replication but otherwise having the same features, is also particularly suitable. It is well known in the art that integration by homologous recombination can be achieved using such so-called suicide vectors, which only have an origin of replication active in Escherichia coli, in actinomycetes (Stimulation of erythromycin yield by integration of a chromosomal DNA fragment including the eryCI gene into the chromosome of S. erythraea, Hanel, F. et al. Biotechnology Letters (1993) 15:105-110; Insertion of plasmids into the chromosome of Streptomyces griseofuscus, Larson, J. L. and Hershberger, C. L. Plasmid (1990) 23:252-256; see also: Denaturation of circular or linear DNA facilitates integrative transformation of Streptomyces coelicolor A3(2): possible relevance to other organisms, Oh, S. H. and Chater, K. F. (1997) J. Bacteriol 179:122-127.)The triketide lactone synthase of the "acceptor" PKS may be composed of loading modules, extension modules and chain-terminating activities drawn from any natural or non-natural Type I PKS, but particularly suitable for this purpose are the components of Type I PKSs for the biosynthesis of erythromycin, rapamycin, avermectin, tetronasin, oleandomycin, monensin, amphotericin and rifamycin, for all of which the gene and modular organisation is known through gene sequence analysis, at least in part. Particularly favourable examples of the loading modules of the donor PKS are those loading modules

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showing a relaxed specificity, for example the loading
module of the avermectin (avr)-producing PKS of
Streptomyces avermitilis; or those loading modules
possessing an unusual specificity, for example the loading
modules of the rapamycin-, FK506- and ascomycin-producing
PKSs, all of which naturally accept a shikimate-derived
starter unit.

Genetically-engineered cells suitable for expression of
hybrid Type I PKS genes may be drawn from any actinomycete
capable of maintaining the vector in either autonomous or
integrated form.   Particularly effective hosts are
Saccharopolyspora erythraea, Streptomyces coelicolor,
Streptomyces avermitilis, Streptomyces griseofuscus,
Streptomyces cinnamonensis, Micromonospora griseorubida,
Streptomyces hygroscopicus, Streptomyces fradiae,
Streptomyces longisporoflavus, Streptomyces lasaliensis,
Streptomyces tsukubaensis, Streptomyces griseus,
Streptomyces venezuelae, Streptomyces antibioticus,
Streptomyces lividans, Streptomyces rimosus and
Streptomyces albus.   These include hosts in which SCP2*-
derived plasmid vectors are known to replicate
autonomously, as for example S. coelicolor, S.
avermitilis and S. griseofuscus; and other hosts such as
Saccharopolyspora erythraea in which SCP2*-derived
plasmids become integrated into the chromosome through
homologous recombination between sequences on the plasmid
insert and on the chromosome; and all hosts which are
integratively transformed by suicide plasmid vectors.

In a further aspect of the present invention, a plasmid
containing "donor" PKS DNA is introduced into a host cell
under conditions where the plasmid becomes integrated into
an acceptor PKS genes on the bacterial chromosome by
homologous recombination, to create a hybrid PKS.   A
preferred embodiment is when the donor PKS DNA includes a
segment encoding a loading module, in such a way that this
loading module becomes linked to the acceptor PKS genes on
the chromosome.   Such a hybrid PKS produces valuable and

novel hybrid polyketide products when cultured under
suitable conditions as described herein. Specifically,
when the loading module of the acceptor PKS is replaced by
the loading module of the avermectin-producing (avr) PKS,
the hybrid polyketide products contain a starter unit
typical of those used by the avr PKS. Thus when the
loading module of the ery PKS is replaced by the avr
loading module, Saccharopolyspora erythraea strains
containing such hybrid PKS are found to produce 14-
membered macrolides containing starter units typically
used by the avr PKS.

It is very surprising and unexpected that the 14-membered
macrolide polyketides produced by such recombinant cells
of S. erythraea are found to include derivatives of
erythromycin A, showing that the several processing steps
required for the transformation of the products of the
hybrid PKS into novel and therapeutically valuable
erythromycin A derivatives are correctly carried out.

A further aspect of the present invention is the
unexpected and surprising finding that transcription of
any of the hybrid Type I PKS genes, whose construction is
described herein, can be specifically increased when the
hybrid genes are placed under the control of a promoter
for a Type II PKS gene linked to a specific activator gene
for that promoter. It is particularly remarkable that
when a genetically engineered cell containing hybrid Type
I genes under such control is cultured under conditions
suitable for polyketide production, significantly
enhanced levels of the hybrid polyketide are produced.
Such specific increases in yield of a valuable polyketide
product are also seen for natural polyketides produced by
a Type I PKS placed under the control of a Type II PKS
promoter and activator gene. In a preferred embodiment,
Type I PKS genes present on an SCP2*-derived plasmid or a
precursor plasmid lacking only the streptomycete origin of
replication, are placed under the control of the actI
promoter derived from the actinorhodin biosynthetic gene

18

cluster of Streptomyces coelicolor, and in which the
vector also contains the structural gene encoding the
specific activator protein Act II-orf 4. The recombinant
plasmid is introduced into bacterial hosts other than
Streptomyces coelicolor chosen from Streptomyces and
related genera, under conditions where either the
introduced PKS genes, or PKS genes already present in the
host strain, are expressed under the control of the actI
promoter.

The recombinant strains produce the desired specific
polyketide product and the activator gene requires only
the presence of the specific promoter in order to enhance
transcriptional efficiency from the promoter. This is
particularly surprising in that activators of the ActII-
orf4 family do not belong to a recognised class of DNA-
binding proteins. Therefore it would be expected that
additional proteins or other control elements would be
required for activation to occur in a heterologous host
not known to produce actinorhodin or a related
isochromanequinone pigment. It is also surprising and
useful that the recombinant strains produce up to 10-fold
more specific polyketide product than when the same PKS
genes are under the control of the natural promoter, and
the specific polyketide product is also produced
precociously in growing culture, rather than only during
the transition from growth to stationary phase. Such
polyketides are useful as antibiotics, anti-cancer agents,
immunosuppressants and for many other purposes in human
and veterinary medicine.

When the genetically engineered cell is Saccharopolyspora
erythraea, the activator and promoter are derived from the
actinorhodin PKS gene cluster and the actI/actII-orf4-
regulated ery PKS gene cluster is housed in the
chromosome; following the site-specific integration of a
plasmid vector, culturing of these cells under suitable
conditions produces up to ten fold more total 14-membered
macrolide product than in a comparable strain not under

19

such heterologous control. When in such a genetically
engineered cell of S. erythraea the PKS genes under this
heterologous control are hybrid Type I PKS genes whose
construction is described herein, then again up to ten-
fold more hybrid polyketide product is obtained compared
to the same hybrid Type I PKS genes not under such
control. Specifically, when the hybrid Type I PKS genes
are the ery PKS genes in which the loading module is
replaced by the avr loading module, a ten-fold increase
is found in the total amounts of novel 14-membered
macrolides produced by the genetically engineered cells
when cultured under suitable conditions as described
herein. The ability of a modular polyketide synthase to
function in a cell-free system has been disclosed, for the
DEBS1-TE system of Saccharopolyspora erythraea (Leadlay,
P. F. Lecture to 9th International Symposium on the
Biology of Actinomycetes, Moscow, July 10-15 (1994) S7-2;
Wiesmann, K. E. et al. Poster presentation P2-02, p 154,
Abstracts of the 9th International Symposium on the
Biology of Actinomycetes, Moscow, July 10-15 (1994);
Wiesmann, K. E. et al. (1995) Chem. and Biol. 2:583-589)
and for the production of 6-deoxyerythronolide B by DEBS1,
DEBS2 and DEBS3 (Pieper et al. (1995) Nature 378:263-
266.). Accordingly, the surprising and unexpected ability
of the actII-orf4 gene to activate the actI promoter in S.
erythraea and to do so more effectively than in its native
host strain, naturally leads to a corresponding impressive
and valuable increase in the amount of active DEBS enzymes
produced by the recombinant S. erythraea strains as
described herein.
The suitable and preferred means of growing the
genetically engineered cells, and the preferred means of
isolating both the natural and the hybrid polyketides are
described more fully in the Examples.
Some embodiments of the invention will now be described
with reference to the accompanying drawings in which:
Fig. 1 gives the chemical formulae of three known

20

polyketides;

Fig. 2A is a diagram showing the functioning of 6-
deoxyerythronolide synthase B (DEBS), a PKS producing 6-
deoxyerythronolide B (6-DEB), a precursor of erythromycin
A;

Fig. 2B shows post-PKS biosynthesis of erythromycins
including the conversion of 6-DEB to erythromycin A;

Fig. 3 is a diagram showing the biosynthesis of rapamycin;

Fig. 4 is a diagram showing the construction of plasmid
pDEL702;

Fig. 5 is a diagram showing the construction of plasmid
pJC3;

Fig. 6 is a diagram showing the construction of plasmid
pCJR1.01; and of the precursor plasmid p20.5 which is now
renamed plasmid pCJR24;

Fig. 7 is a diagram showing the construction of plasmid
pCJR110 which is now renamed plasmid pCJR29;

Fig. 8 is a diagram showing the construction of plasmid
pNTEP2;

Fig. 9 is a diagram showing the construction of plasmid
pRMTE and pCJRTE; the latter plasmid is now renamed
pCJR30;

Fig. 10 a and b is a diagram showing the construction of
plasmid pIG1;

Fig. 11 is a diagram showing the construction of plasmid
pND20;

Fig. 12 is a diagram showing the construction of plasmid
pKW15; this includes DNA encoding a loading module, a
first extender module, and the chain-terminating
thioesterase, capable of receiving modules;

Fig. 13 is a diagram showing the construction of plasmid
pAR33;

Fig. 14 is a diagram showing the construction of plasmid
pAR8;

Fig. 15 is a diagram showing the construction of plasmid
pE1A2TE;

Fig. 16 is a diagram showing the construction of plasmid

pKS22;

Fig. 17a is a diagram showing the construction of plasmid pIB018;

Fig. 17b is a diagram showing the construction of plasmid pIB017;

Fig. 18 is a diagram showing the construction of plasmid pIB015 and plasmid pIB016;

Fig. 19 is a diagram showing the construction of plasmid pJLK15;

Fig. 20 is a diagram showing the construction of plasmid pJLK18;

Fig. 21 is a diagram showing the construction of plasmid pJLK21;

Fig. 22 is a diagram showing the construction of plasmid pKR1-0;

Fig. 23 is a diagram showing the construction of plasmid pKETO;

Fig. 24 is a diagram showing the construction of plasmid pMO7;

Fig. 25 is a diagram showing the construction of plasmid pCJR26;

Fig. 26 is a diagram showing the construction of plasmid pC-ATX;

Fig. 27 is a diagram showing the construction of plasmid pC-AT12;

Fig. 28 is a diagram showing the construction of plasmid pCJR49;

Fig. 29 is a diagram showing the construction of plasmid pCART11;

Fig. 30 is a diagram showing the construction of plasmid pARE24;

Fig. 31 is a diagram showing the construction of plasmid pARL3;

Fig. 32 is a diagram showing the construction of plasmid pAVLD;

Fig. 33 shows the integration of pAVLD into the genome of S.erythraea NRRL2338; and

Fig. 34 shows the integration of pAVLD into the genome of
S.erythraea TER43.

The present invention will now be illustrated, but is not
intended to be limited, by means of some examples.
Use was made of the following media and solutions.

Sucrose-Succinate defined medium

sucrose	69g
KNO_3	10g
succinic acid	2.36g
KH_2PO_4	2.7g
$MgSO_4.7H_2O$	1.2g
$ZnCl_2$	10mg
$MnCl_2.4H_2O$	6.2g
$CuCl_2.2H_2O$	0.53mg
$CoCl_2$	0.55mg
$FeSO_4.7H_2O$	2.5mg
$CaCl_2.2H_2O$	38mg
milli-Q water	to 1l
KOH	To pH 6-6.4

YEME

Tap water medium

sucrose	340g
glucose	5g
yeast extract	3g
tryptone	5g
peptone	5g
yeast extract	2.5g
malt extract	3g
EDTA	36mg
glucose	10g
tap water	to 1.0 l
KOH	to pH7.1

after sterilisation: 2.5M $MgCl_2$ 2ml

Trace elements solution: $ZnCl_2$, 40mg/l; $FeCl_3.6H_2O$,

23

200mg/l; $CuCl_2.2H_2O$, 10 mg/l; $MnCl_2.4H_2O$, 10mg/l; $Na_2B_4O_7$
$.10H_2O$), 10mg/l; $(NH_4)_6MO_7O_{24}.4H_2O$, 10mg/l;

BW1 medium

$CaCO_3$	2	g
Difco tryptone	2.5	g
soy flour	5	g
Difco yeast extract	5	g
soluble starch (Sigma)	20	g

pH 7.2

K_2HPO_4	1.2	g
$MgSO_4.7H_2O$	1.2	g
$FeSO_4.7HO_2O$	0.012	g
$MnSO_4$	0.0012	g
$ZnSO_4.7H_2O$	0.0012	g
Tap water	to 1.0	l

BW2 Medium

$CaCO_3$	7 g
soy flour	5 g
Difco yeast extract	5 g
soluble starch (Sigma)	80 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	1 g

10 ml of a trace elements solution

Made up to 1 litre with distilled water.   pH adjusted to
7.2.

Example 1
Construction of strain Saccharopolyspora erythraea JC2

An S. erythraea host cell, genetically engineered to
remove all of the native eryA genes which encode the
erythromycin-producing type I PKS, except for the region
of eryAIII DNA encoding the chain-terminating
thioesterase, was constructed by homologous recombination

starting from S. erythraea NRRL2338. S. erythraea
NRRL2338 is a wild-type erythromycin-producing strain
obtained from the Northern Regional Research
Laboratories, Peoria, Illinois, USA, under the above
designation. The ery cluster is made up of the PKS
genes, flanked by other genes involved in later stages of
erythromycin biosynthesis, including those involved in
glycosylation, hydroxylation and methylation.
Plasmid pDEL was constructed as follows (Figure 4). The
1.4 kbp SmaI segment containing the start codon of eryAI
was cloned into pUC18 to give p612SL, the segment was
excised as a BamHI-SacI fragment using the multiple
cloning sites of pUC18, and subcloned into a derivative
of plasmid pT7-18 (Roberts, G. A. et al. Eur. J. Biochem.
(1993) 214:305-311)) containing the SacI/KpnI fragment
of eryAIII that encodes the C-terminus of DEBS3 from
which a BglII-SacI fragment had been excised. The
identity of plasmid pDEL was confirmed by restriction
analysis.
Plasmid pDEL was digested with BamH1 and treated with
calf intestinal alkaline phosphatase, and ligated to
plasmid pIJ702 (Katz, E. et al. J. Gen. Microbiol. (1983)
129:2703-2714) which had been linearised with BglII. The
resulting mixture contains the desired plasmid pDEL702
(Figure 4).
Protoplasts of S. erythraea NRRL2338 (Yamamoto, H. et al.
J. Antibiot. (1986) 39:1304-1313) were transformed with
10 μg pDEL702 and stable thiostrepton resistant colonies
were isolated. Individual colonies were selected and
subcultured four times in non-selective liquid medium
(tryptic soy broth) followed by preparation and
regeneration of protoplasts. Thiostrepton sensitive
colonies were isolated and characterised by restriction
analysis and Southern hybridisation. One such colony was
designated JC2. S. erythraea strain JC2 has been
deposited at the National Collection of Industrial and
Marine Bacteria, 23 St Machar Drive, Aberdeen, Scotland

25

AB2 1RY, under the designation NCIMB 40802.

Example 2

Construction of strain Saccharopolyspora erythraea JC3

An S. erythraea host cell, genetically engineered which
contains a derivative plasmid of pCJR29 integrated in the
chromosome useful for the expression of homologous and
heterologous genes was constructed by homologous
recombination starting from S. erythraea JC2 (Figure 5).

Plasmid pCJR29K was constructed as follows (Figure 5),
the 1.4 kbp SacI-SphI restriction fragment containing the
kanamycin resistance gene from plasmid pIJ6021 (Takano,
E. et al. Gene (1995) 166:133-137) was cloned into SacI-
SphI digested pUC18 to produce pKAN, this plasmid was
digested with PvuII and the 1.7 kbp fragment containing
the kanamycin resistance gene was cloned into EcoRV
digested pCJR29 to produce plasmid pCJR29K.

Plasmid pJC3 was constructed as follows (Fig 5), the 6.2
kbp SpeI-XbaI restriction fragment from pNCO62 (Gaisser,
S. et al. Mol. Gen. Genet. (1997) in press) was cloned
into Xba I-digested pCJR29K to produce pJC3.

Protoplasts of S. erythraea JC2, prepared as described
for S. erythraea NRRL2338, were transformed with 10 μg
pJC3 and stable kanamycin (100 μg/ml) resistant colonies
were isolated. Individual colonies were isolated and
characterised by restriction analysis and Southern
hybridisation. One such colony was designated S.
erythraea JC3.

Example 3

Construction of strain S. erythraea JC103 (NRRL2338/pNHE)

To obtain an S. erythraea strain that overexpresses

26

DEBS1, DEBS2 and DEBS3, the construction of intermediate
plasmids was carried out as follows.

Construction of pARLD

The 1.6 kbp DNA segment encoding the loading domain of
the erythromycin polyketide synthase from nucleotide 1 to
1680 was amplified by PCR employing the CloneAmp
procedure (Raschtian, A. et al. Anal. Biochem. (1992)
91: 91-97) with the following two oligonucleotides as
primers:
5'-ACGCGUACUAGUCCGATTAATTAAGGAGGACCATCATGGCGGACC
TGTCAAAGCTC-3' and
5'-AUGGAGAUCUCUCCGCTAGCGGTTCGCCGGGCGCCGCTTCGTTGGTCCGC
GCGCGGGTTTCCC-3'
and using as template the DNA of plasmid pNTEP2.
Approximately 30-60 ng of the PCR product (1.6 kbp) is
digested with uracil DNA glycosylase for 30 minutes at
37°C in the presence of 25 ng of pAMP18 vector DNA (Gibco
BRL), the mixture is cooled on ice and used to transform
E. coli TG1recO and individual colonies are checked for
their plasmid content. The desired plasmid is identified
by its restriction map and is designated pARLD

Construction of pNHE

A 1.6 kbp fragment of plasmid pARLD is excised using PacI
and NheI, purified by gel electrophoresis, and ligated to
plasmid pCJR24 which had been cut with PacI and XbaI.
The ligation mixture is transformed into E. coli DH10B
(Gibco BRL) and individual colonies, grown in the
presence of ampicillin (100 μg/ml), are checked for their
plasmid content. The desired plasmid is identified by its
restriction map and is designated pNHE.

Construction of S. erythraea JC103 (NRRL2338/pNHE)

27

Approximately 5 µg pNHE, isolated from E. coli DH10B
(pNHE) is used to transform S. erythraea NRRL2338
protoplasts and stable thiostrepton resistant colonies
are selected. One of this colonies is selected and total
DNA is prepared for Southern hybridisation analysis, to
confirm that the plasmid has integrated specifically into
the chromosomal copy of the eryAI gene in the area that
encodes the N-terminal loading domain. This strain is
designated S. erythraea JC103 (NRRL2338/pNHE).

Example 4
Construction of the Recombinant Vector pCJR101

pCJR101 (Figure 6) is a shuttle plasmid constructed to be
used for expression of PKS genes in actinomycetes. It
includes a ColEI replicon to allow it to replicate in E.
coli, an SCP2* low copy number Streptomyces replicon
(Bibb, M. J. and Hopwood, D. A. J. Gen. Microbiol.
(1981) 126:427) and the actII-orf4 activator gene from
the act cluster which activates transcription from the
act promoter during the transition from growth phase to
stationary phase in the vegetative mycelium. It is
constructed as follows: an approximately 970 bp DNA
fragment from pMF1015 (containing the actII-orf4
activator gene) (Fernandez-Moreno, M. A. et al. Cell
(1991) 66:769-780) is amplified by PCR, using as primers
the synthetic oligonucleotides:
5'-ACT AGT CCA CTG CCT CTC GGT AAA ATC CAG C-3' and 5'-
CTT AAG AGG GGC TCC ACC GCG TTC ACG GAC-3', which also
introduces flanking SpeI and AflII restriction sites.
This fragment is introduced into the end-repaired AatII
site of plasmid pUC19 to yield plasmid p18.14 (renamed
pCJR18). An approximately 215 bp DNA fragment is
amplified from pMV400 which contains the bidirectional
promoter pair PactIII/PactI) (Parro, V. et al. Nucl.
Acids Res. (1991) 19:2623-2627), using as primers the
synthetic oligonucleotides 5'-ACA TTC TCT ACG CCT AAG TGT

28

TCC CCT CCC TGC CTC-3' and 5'-GTG ATG TAT GCT CAT ATG TGT
CCT CCT TAA TTA ATC GAT GCG TTC GTC CGG TG-3', which also
introduces flanking NdeI and AflII sites. The PCR
product is digested with NdeI and AflII and ligated with
the plasmid p18.14 (pCJR18) previously cut with NdeI and
AflII, to generate plasmid p19.4 (renamed pCJR19). A 1.1
kbp HindIII-SphI fragment containing the tsr gene, which
confers resistance to thiostrepton, is obtained by PCR
from plasmid pIJ922 (Lydiate, D. J. et al. Gene (1985)
35:223-235) as template, using as primers the
oligonucleotides 5'-TGA ACA CCA AGC TTG CCA GAG AGC GAC
GAC TTC CCC-3' and 5'-GAC AGA TTG CAT GCC CTT CGA GGA GTG
CCC GCC CGG-3' which also introduces flanking HindIII and
SphI sites. The PCR·product is digested with HindIII and
SphI and ligated with plasmid p19.4 (pCJR19) cut with
HindIII and SphI to obtain plasmid p20.5 (pCJR24). The
plasmid pIJ922 is digested with BamHI and SstI and the
fragment containing a portion of the fertility locus and
the origin of replication (Lydiate, D. J. et al. Gene
(1985) 35:223-235) is ligated into pUC19 digested with
BamHI and Sst I to generate the bifunctional plasmid
p16/2.2 (renamed pCJR16) (14.7 kbp).   Plasmid p20.5
(pCJR24) is digested with SalI and SphI, the two larger
fragments from the digest are purified by gel
electrophoresis, and combined in a four-component
ligation with plasmid 16/2.2 (pCJR16) which has been
digested with XhoI and SphI. The ligation mixture is
used to transform Streptomyces lividans and colonies are
selected in the presence of thiostrepton. One such
colony is shown to contain the desired plasmid pCJR101
(approx. 12.4 kbp), identified by its restriction
pattern.

Example 5
Construction of plasmid pCJR29 (renamed from pCJR110)

The construction of plasmid pCJR29 (pCJR110) is

illustrated in Figure 7. A 1.1 kbp HindIII-XhoI fragment
containing the tsr gene, which confers resistance to
thiostrepton, is obtained by PCR from plasmid pIJ922 as
template, using as primers the oligonucleotides 5'-TGA
ACA CCA AGC TTG CCA GAG AGC GAC GAC TTC CCC-3' and 5'-GAC
AGA TTC TCG AGC CTT CGA GGA GTG CCC GCC CGG-3' which also
introduces flanking HindIII and XhoI sites. The PCR
product is digested with HindIII and XhoI and ligated
with plasmid 16/2.2 (pCJR16) which has been digested with
HindIII and XhoI, to generate plasmid 22.1 (pCJR25) .
Plasmid p22.1 (pCJR25) is digested with HindIII and SphI
and ligated with plasmid p19.4 (pCJR19) which has been
digested with HindIII and SphI, to produce the desired
plasmid pCJR29 (pCJR110) (approx. 12.4 kbp), identified
by its restriction pattern. Plasmid pCJR29 (pCJR110)
differs from pCJR101 in the orientation of the tsr gene,
the actII-orf4 gene and the actI/actIII promoter, with
respect to the SCP2*-derived origin of replication.

Example 6
Construction of plasmid pRM52

Plasmid pRM52 is a derivative of plasmid pRM5 (McDaniel,
R. et al. Science, (1993) 262:1546-1550). pRM5 was first
linearised by digestion with NdeI, end-repaired and then
religated to produce pRM51. pRM51 was cut with PacI and
NsiI and the large PacI-NsiI fragment was isolated and
ligated to a short double-stranded oligonucleotide linker
containing an NdeI site and constructed from the
synthetic oligonucleotides 5'-TAAGGAGGACACATATGCA-3' and
5'-TAATTCCTCCTGTGTAT-3' which were annealed together.
The ligation mixture was transformed into E. coli TGIrecO
and isolated colonies were screened for their plasmid
content. The desired plasmid (19.6 kbp) was identified
by its restriction map and was designated pRM52.

Example 7

30

Construction of plasmid pNTEP2

Plasmid pNTEP2 contains the entire open reading frame for
the chimaeric DEBS1 plus thioesterase gene, with a unique
NdeI site at the start codon and unique XbaI and HindIII
sites immediately 3' of the stop codon. It is
constructed via several intermediate plasmids as follows
(Figure 8):

Construction of plasmid pTENCO11
A library of total DNA from S. erythraea TED8 (Cortes, J.
et al. Science (1995) 268: 1487-1489) was constructed in
the vector _DASH II (Stratagene) and probed with eryA
gene fragments. One recombinant bacteriophage designated
λ-4B had an insert extending from 700 bp upstream of the
eryAI start codon to the thiostrepton resistance gene of
the integrated plasmid in S. erythraea TED8.
The λ-4b DNA was digested with NcoI and the 12 kbp NcoI
fragment was end-repaired and ligated into SmaI-cut
pUC18 and transformed into E. coli TG1recO. Individual
colonies were screened for their plasmid content and one
plasmid bearing the NcoI insert was selected and
designated pTENCO11.
Construction of plasmid pNK8
A 4.0 kb KpnI fragment extending from 1.4 kbp upstream of
the correct eryAI start codon as previously determined
(Caffrey, P. et al. FEBS Letters (1992) 304:225-228), to
2.6 kbp inside the eryAI gene of S. erythraea, was
excised from plasmid pBK25 (Bevitt, D. J. et al. Eur. J.
Biochem. (1992) 204:39-49) and cloned into pUC18 to
obtain plasmid pBK6.12. DNA of this plasmid was used as
the template for a PCR reaction to obtain a 360 bp
product in which a unique Nde I site is created at the
start codon of eryAI and a unique SmaI site is created at
the other end of the PCR product. The oligonucleotides
used were 5'-CCC ATA TGG CGG ACC TGT CAA AGC-3' and 5'-
ATT GCG CGC CCT GGC CCG GGA A-3'. The product was end-

repaired and ligated into SmaI cut pUC18, and transformed
into E. coli TG1recO.
Individual colonies were screened for their plasmid
content and one plasmid bearing the insert in an
orientation such that the SmaI site was adjacent to the
KpnI site of the polylinker was selected and designated
plasmid pNDE6. Plasmid pNDE6 was digested wth SmaI and
KpnI, and ligated with a 2.3 kbp fragment of the eryAI
gene obtained by digestion of plasmid pBK6.12 with SmaI
and KpnI. The ligation mixture was used to transform E.
coli TG1recO and individual colonies were screened for
their plasmid content. A plasmid containing the desired
2.6 kbp NdeI-KpnI fragment was isolated and designated
plasmid pNDE7. The NdeI-KpnI insert was excised from
plasmid pNDE7 and ligated into plasmid pT7-18, previously
digested with NdeI and KpnI. Plasmid pT7-18 is a
derivative of plasmid pT7-7 (Tabor, S. and Richardson,
C.C. Proc. Natl. Acad. Sci. USA (1985) 82:1074-1078) in
which the polylinker is replaced by the polylinker from
pUC18. The ligation mixture was used to transform E.
coli TG1recO and individual colonies were screened for
their plasmid content and one plasmid containing the
desired 2.6 kbp NdeI-KpnI insert was selected and
designated pNK8.

Construction of plasmid pNTE5

Plasmid pNK8 was transformed into a methylation-deficient
strain of E. coli ET12567 (MacNeil, D.J. et al. Gene
(1992) 111:61-68) and the plasmid pNK8 was isolated from
this strain and digested with ClaI . An 11 kbp ClaI
fragment obtained by digestion of pTENC011 was ligated
into the digested pNK8 and transformed into E. coli
TG1recO. Individual colonies were screened for their
plasmid content and one plasmid, in which the 11 kbp
insert was correctly oriented to regenerate the reading
frame of eryAI, was selected and designated pNTE5.

32

Construction of plasmid pNTEP2

A ClaI-EcoRI polylinker, bearing unique restriction sites
for XbaI and for HindIII was constructed, from the
following complementary synthetic oligonucleotides:
5'-AATTCATAGTCTAGAAGCTTAT-3'
and
5'-CGATAAGCTTCTAGACTATG-3'
The polylinker was ligated into plasmid pNTE5, which had
been digested with ClaI and EcoRI to remove a 2.3 kbp
ClaI-EcoRI fragment. The ligation mixture was used to
transform E. coli TG1recO and individual colonies were
screened for their plasmid content. One plasmid
containing the polylinker was identified and designated
pNTEP2.

Example 8
Construction of plasmid pRMTE

Plasmid pNTEP2 (14 kbp) was digested with NdeI and XbaI
and the insert was purified by sedimentation on a sucrose
gradient. The purified insert was ligated into plasmid
pRM52 (19.6 kbp) (Example 4) which had been digested with
NdeI and XbaI, and the vector purified by sedimentation
on a sucrose gradient. The ligation mixture was used to
transform E. coli and individual colonies were checked
for their plasmid content. The desired plasmid pRMTE
(31.5 kbp) was identified by its restriction pattern
(Figure 9).

Example 9
Construction of plasmid pCJRTE (also named pCJR30)

Plasmid pNTEP2 (Example 5) is digested with NdeI and XbaI
and the insert is purified by sedimentation on a sucrose
gradient. The purified insert is ligated into plasmid
pCJR101 (12.4 kbp) which has been digested with NdeI and

33

XbaI, and purified by sedimentation on a sucrose
gradient. The ligation mixture is used to transform E.
coli DHB10 and individual colonies are screened for their
plasmid content. The desired plasmid pCJRTE (pCJR30)
(24.3 kbp) is identified by its restriction pattern
(Figure 9).

Example 10
Construction of S. avermitilis ATCC 31272/pCJRTE (pCJR30)
and production of triketide lactone ("TKL") derivatives
therewith.
(i) Construction
Approximately 5 μg of plasmid pCJRTE (pCJR30) is
transformed into protoplasts of S. avermitilis ATCC 31272
and stable thiostrepton resistant colonies are isolated.
Several such colonies are analysed for their content of
plasmid DNA. A colony containing a plasmid whose
restriction map shows it to be identical to pCJRTE
(pCJR30), is designated S. avermitilis ATCC 31272/pCJRTE
(pCJR30).

(ii) Production of (Ac)-TKL and TKL

S. avermitilis ATCC 31272/pCJRTE (pCJR30) is inoculated
into medium BW1 containing 50 μg/ml thiostrepton, and
allowed to grow for four days at 28-30%C. After this
time, 15 ml of the cell suspension is used to inoculate
150 ml of liquid medium BW2 containing 50_g/l
thiostrepton, and allowed to grow for 6 days. After this
time the cells are removed by centrifugation, washed with
water, and the supernatants are combined and extracted
three times with ethyl acetate (250 ml). The combined
ethyl acetate extracts are washed with an equal volume of
saturated sodium chloride, dried over anhydrous sodium
sulphate and the ethyl acetate is removed by evaporation
under reduced pressure. Samples of the residue are taken
up in a minimal quantity of diethyl ether, filtered

34

through a plug of silica, and analyzed by GC, which
reveals the presence of both (Ac)-TKL and TKL (Formula
II: $R_1=R_2=Me$; $R_3=Me$ for (Ac)-TKL, and Et for TKL), with
identical retention times to authentic synthetic samples.

Example 11
Construction of plasmids pIG1 and pIG101

Plasmids pIG1 and pIG101 each consist of an SCP2*-derived
plasmid containing a hybrid Type I PKS gene comprising
the avr loading module in place of the ery loading
module, the first two extension modules of the ery PKS
and the thioesterase of the ery PKS. These are
constructed via several intermediate plasmids as follows
(Figure 10).

Construction of plasmid pVE 3.4

Plasmid pVE1446 which contains a portion of the
avermectin (avr) PKS genes was obtained from E. coli
strain ATCC 68250 (MacNeil, D. J. et al. Ann. N. Y. Acad.
Sci. (1994) 721:123-132). Plasmid pVE1446 was digested
with BamHI and the 7.6 kbp fragment between coordinates
32.15 and 3.40 (MacNeil, D. J. et al. Ann. N. Y. Acad.
Sci. (1994) 721:123-132) was purified by gel
electrophoresis and recircularised. The mixture
contained the desired plasmid pVE3.4 which was isolated
after transformation of E. coli strain TG1recO.

Construction of plasmid pNCO12

Plasmid pBK25 (Bevitt, D. J. et al. Eur. J. Biochem.
(1992) 204:39-49) was digested with NcoI and the 12 kbp
fragment was end-repaired and ligated into plasmid pUC18
which had been linearised with SmaI. The ligation mixture
was transformed into E. coli TG1 recO and individual
colonies were checked for their plasmid content. The

SUBSTITUTE SHEET (RULE 26)

35

desired plasmid pNCO12 was identified by its restriction
pattern.

Construction of plasmid pCRabc

Plasmid pCRabc (Figure 10) was constructed as follows.
Three separate PCR reactions were conducted: First, 20
pmol each of synthetic oligonucleotides A1 (5'-CTC GTC
GGT GGC TTT GCG-3') and A2 (5'-CCC GGG AAA AAC GAA GAC
TAG TGG CGC GGA CGG CCG-3') were used to amplify a 1.0
kbp product from 100 ng pNCO12 template. The PCR product
was end-repaired, phosphorylated and cloned into SmaI-
cut pUC18 to obtain plasmid pCRa. Secondly, 20 pmol each
of synthetic oligonucleotides C1 (5'-CAC GCG CAG CGC GGC
GGA-3') and C2 (5'-CGAA CCG CTA GCG GTC GTC GCG ATG GCC
T-3') were used to amplify a 1.5 kbp product from 100 ng
pNCO12 template. The product was end-repaired,
phosphorylated and cloned into SmaI-cut pUC18 to obtain
plasmid pCRc. Thirdly, 20 pmol each of synthetic
oligonucleotides B1 (5'-
GTGGCCCGGCCGTCCGCGCCACTAGTCTTCGTTTTT-3') and B2 (5'-AAC
AGCTAGCGGTTCGTCCGCCGCTGCCGTGCC-3') were used to amplify a
1.4 kbp product from 100 ng pVE3.4 template. The product
was end-repaired, phosphorylated and cloned into SmaI-cut
pUC18 to obtain plasmid pCRb.

Plasmid pCRa was digested with HindIII and SpeI and the
1.0 kbp insert was ligated with plasmid pCRb previously
digested with HindIII and SpeI, to obtain plasmid pCRab.
Plasmid pCRc was digested with NheI and EcoR1 and the 1.5
kbp insert was ligated with plasmid pCRab previously
digested with NheI and EcoR1 to obtain plasmid pCRabc.

Construction of plasmid pNEWAVETE

Plasmid pCRabc was digested with MfeI and SfiI and the
DNA fragment containing the loading domain of the avr PKS

was purified by gel electrophoresis and ligated with
plasmid pNTEP2 which had been digested with MfeI and SfiI
and the larger fragment purified by gel electrophoresis.
The ligation mixture was transformed into E. coli TG1
recO and individual colonies were checked for their
plasmid content. The desired plasmid pNEWAVETE (13.7
kbp) was identified by its restriction pattern.

Construction of plasmid pIG1

Plasmid pNEWAVETE was digested with NdeI and XbaI and the
insert was purified by sedimentation on a sucrose
gradient. The purified insert was ligated into plasmid
pRM52 (19.6 kbp) which had been digested with NdeI and
XbaI, and the vector purified by sedimentation on a
sucrose gradient. The ligation mixture was used to
transform E. coli and individual colonies were checked
for their plasmid content. The desired plasmid pIG1 was
identified by its restriction pattern.

Construction of plasmid pIG101

Plasmid pNEWAVETE is digested with NdeI and XbaI and the
insert is purified by sedimentation on a sucrose
gradient. The purified insert is ligated into plasmid
pCJR101 (Example 4) which has been digested with NdeI and
XbaI, and purified by gel electrophoresis. The ligation
mixture is used to transform E. coli DHB10 and individual
colonies are screened for their plasmid content. The
desired plasmid pIG101 is identified by its restriction
pattern.

Example 12
Construction of S. coelicolor CH999/pIG1 and production
of TKL derivatives.
(i) Construction
Plasmid pIG1 which had been isolated from E. coli ET12567

(MacNeil. D. J. et al. Gene (1992) 111:61-68) was used to transform protoplasts of S. coelicolor CH999 and stable thiostrepton resistant colonies were isolated. Individual colonies were checked for their plasmid content and the presence of plasmid pIG1 was confirmed by its restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-pent)TKL using S. coelicolor CH999/pIG1

S. coelicolor CH999/pIG1 was inoculated into 100 ml YEME medium containing 50 μg/ml thiostrepton and allowed to grow for five days at 28-30°C. After this time the broth was filtered to remove mycelia. The broth was extracted three times with quarter volumes of ethyl acetate and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and the ethyl acetate was removed under reduced pressure. The residue was taken up in ethyl acetate and filtered through a plug of silica, the ethyl acetate was again removed and the residue was taken up in diethyl ether and subjected to flash chromatography on a column of silica gel eluted with diethyl ether. A fraction containing (s-pent)-TKL and (i-but)-TKL was separated from a fraction containing TKL, with minor amounts of (Ac)-TKL in a third fraction. The compounds were identified by their co-migration with authentic standards on GC analysis (25m column, programmed for 2 minutes at 70°C, then ramped to 250°C over 24 minutes. The retention times for (s-pent)-TKL, (i-but)-TKL, TKL and (Ac)-TKL were 14.9, 13.6, 12.9 and 11.9 minutes respectively. GC, electrospray MS and 1H-NMR were used to show that the major component (50-60%) was TKL.

Example 13
Construction of S. coelicolor CH999/pIG101 and production of TKL derivatives

38

(i) Construction

Plasmid pIG101 which has been isolated from E. coli
ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) is
used to transform protoplasts of S. coelicolor CH999 and
stable thiostrepton resistant colonies were isolated.
Individual colonies are checked for their plasmid content
and the presence of plasmid pIG101 is confirmed by its
restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-
pent)TKL using S. coelicolor CH999/pIG101

S. coelicolor CH999/pIG101 is inoculated into YEME medium
containing 50 μg/ml thiostrepton and allowed to grow for
five days at 28-30°C. The broth is extracted three times
with quarter volumes of ethyl acetate and the combined
ethyl acetate extracts are dried over anhydrous sodium
sulphate, and the ethyl acetate is removed under reduced
pressure. The residue was treated as in Example 12 and
gave similar results.

Example 14
Construction of S. avermitilis ATCC31272/pIG1 and
production of TKL derivative
(i) Construction

Plasmid pIG1 which had been isolated from E. coli ET12567
(MacNeil, D. J. et al. Gene (1992) 111:61-68) was
transformed into protoplasts of S. avermitilis ATCC31272
and stable thiostrepton resistant colonies were isolated.
Individual colonies were checked for their plasmid
content and the presence of plasmid pIG1 was confirmed by
its restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-
pent)TKL using S. avermitilis ATCC31272/pIG1

S. avermitilis ATCC31272/pIG1 was first inoculated into
medium BW1 containing 50 μg/ml thiostrepton, and allowed
to grow for four days at 28-30°C. After this time 20 ml
of the broth is used to seed 150 ml of medium BW2
containing 50 μg/ml of thiostrepton.

The inoculated organism was then allowed to grow for 10-
12 days. The broth was filtered to remove mycelia, and
extracted three times with quarter volumes of ethyl
acetate and the combined ethyl acetate extracts were
dried over anhydrous sodium sulphate, and the ethyl
acetate was removed under reduced pressure, to give about
10 mg crude product per litre. The residue was dissolved
in ethyl acetate, passed through a plug of silica, and
the solvent was removed. The residue was dissolved in
diethyl ether and subjected to flash chromatography on a
silica column (1 cm x 15 cm) eluted with diethyl ether,
and fractions of 10 ml each were collected and assayed by
GC. The diethyl ether was evaporated to leave about 10
mg of oily residue containing triketide lactones. The
major component (50-60%) was (s-pent)-TKL, with (i-but)-
TKL, TKL and (Ac)-TKL also present (i.e. compounds of
formula II with $R_1=R_2=Me$, and $R_3=1$-methylpropyl ((s-pent)-
TKL), i-Pr((i- But)-TKL), Et(TKL) and Me((Ac)-TKL).

Example 15
Construction of S. avermitilis ATCC31272/pIG101 and
production of TKL derivatives

(i) Construction

Plasmid pIG101 which has been isolated from E. coli
ET12567 (MacNeil, D. J. et al. Gene (1992) 111:61-68) is
transformed into protoplasts of S. avermitilis ATCC31272
and stable thiostrepton resistant colonies are isolated.
Individual colonies are checked for their plasmid content
and the presence of plasmid pIG101 is confirmed by its

40

restriction pattern.

(ii) Production of TKL, (Ac)TKL, (i-but)TKL and (s-
pent)TKL using S. avermitilis ATCC31272/pIG101

S. avermitilis ATCC31272/pIG101 is first inoculated into
medium BW1, described above and allowed to grow for 10-12
days. Isolation of products as in the previous example
gives a fraction containing (s-pent)-TKL and (i-but)-TKL,
a fraction containing TKL, and a third fraction with
minor amounts of (Ac)-TKL. The compounds are identified
by their co-migration with authentic standards on GC
analysis.

Example 16
Construction of S. erythraea JC2/pIG1 and production of
TKL derivatives

(i) Construction

Approximately 5 μg of plasmid pIG1 is transformed into
protoplasts of S. erythraea JC2 and stable thiostrepton
resistant colonies are isolated. From several such
colonies, total DNA is obtained and analysed by Southern
hybridisation, to confirm that the plasmid has integrated
specifically into the portion of the eryAIII gene that
encodes the C-terminal thioesterase/cyclase, by
homologous recombination.

(ii) Production of triketide lactones using S. erythraea
JC2/pIG1

S. erythraea JC2/pIG1 is inoculated into tap water medium
containing 50 μg/ml thiostrepton and allowed to grow for
four days at 30°C. After this 20 ml of the mycelium is
used to seed 500 ml of sucrose-succinate medium
containing 50 μg/ml thiostrepton, in a 2L flask with a

SUBSTITUTE SHEET (RULE 26)

single spring to reduce clumping, shaken at 280 rpm.
After between 3.5 and 6 days, the broth is filtered to
remove mycelia and then extracted three times with a
quarter volume of ethyl acetate. The combined ethyl
acetate extracts are dried over anhydrous sodium sulphate
and solvent removed by evaporation. Analysis of the
product mixture using GC and electrospray MS revealed
that of a total of 5-6 mg/L of triketide lactone
products, the major component was (s-pent)-TKL (about 1.5
mg/L), with other components present being (i-but)-TKL,
TKL and a minor amount of (Ac)-TKL.

Example 17
Construction of S. erythraea JC2/pIG101 and production of
TKL derivatives

(i) Construction
Approximately 5 μg of plasmid pIG101 is transformed into
protoplasts of S. erythraea JC2 and stable thiostrepton
resistant colonies are isolated. From several such
colonies, total DNA is obtained and analysed by Southern
hybridisation, to confirm that the plasmid has integrated
specifically into the portion of the eryAIII gene that
encodes the C-terminal thioesterase/cyclase, by
homologous recombination.

(ii) Production of triketide lactones using S. erythraea
JC2/pIG101

The same procedure as in Example 16 (ii) was followed.
Analysis of the product mixture using GC and electrospray
MS revealed that of a total of 5-6 mg/L of triketide
lactone products, the major component was (s-pent)-TKL
(about 1.5 mg/L), with other components present being (i-
but)-TKL, TKL and a minor amount of (Ac)-TKL.

Example 18

42

Construction of plasmid pND20

This was accomplished in two stages:

(i) Construction of plasmid pHISAVE

Plasmid pNEWAVETE was digested with EcoRI and HindIII and
the vector was purified by gel electrophoresis. A
synthetic oligonucleotide double-stranded insert encoding
a 6-histidine tag and possessing these sites at either
end (shown below) was ligated to the vector.

(5'-AATTCACATCACCATCACCATCACTAGTAGGAGGTCTGGCCATCTAGA-3')
(3'-GTAGTGGTAGTGGTAGTGATCATCCTCCAGACCGGTAGATCTTCGC-5')

The ligation mixture was used to transform E.coli DH10B
and individual colonies were screened for their plasmid
content. The desired plasmid, pHISAVE was identified by
its restriction pattern.

(Ii) Construction of plasmid pND20

Plasmid pHISAVE was digested with NdeI and XbaI and the
insert was ligated into pCJR24 digested with NdeI and
XbaI. The ligation mixture was used to transform DH10B
and individual colonies were screened for their plasmid
content. The desired plasmid, pND20 was identified by
its restriction pattern.

Example 19

(i) Construction of S. erythraea JC3/pND20

Plasmid pND20 which has been isolated from E. coli
ET12567 is used to transform protoplasts of S. erythraea
JC3 and stable thiostrepton colonies are isolated.

SUBSTITUTE SHEET (RULE 26)

43

(ii) Production of TKL and (Ac)TKL

S. erythraea JC3/pND20 is inoculated into tap water
medium containing 50μg/ml thiostrepton and allowed to
grow for four days at 30°C. 20ml of this is used to
inoculate 500ml of sucrose-succinate medium containing
50μg/ml thiostrepton, in a 2L flask with a single spring
to reduce clumping and shaken at 280rpm. After between
3.5 and 6 days, the broth is filtered to remove mycelia
and then extracted three times with a quarter-volume of
ethyl acetate. The combined ethyl acetate extracts are
dried over anhydrous sodium sulphate and the solvent is
removed by evaporation. Analysis of the product mixture
using GC and electrospay MS reveals that of a total of
about 20mg/L of triketide lactone products, about ninety
percent consisted of TKL and the remainder was Ac(TKL).

Example 20
Construction of plasmid pKW15

Plasmid pKW15 is a pT7-derived vector containing an
insert comprising the loading module, the first extension
module and the thioesterase of the ery PKS, suitable for
subcloning into an SCP2*-based vector to obtain
expression of a diketide synthase gene; and also suitable
for insertion of heterologous DNA containing one or more
intact modules. Plasmid pKW15 is obtained via several
intermediate plasmids as follows (Figure 12).

Construction of plasmid pKW11
Plasmid pNTEP2 (Example 5) was digested with BglII, the
sticky ends were filled in and religated, to produce
plasmid pKW11. The insert in plasmid pKW11 consists of a
chimaeric eryAI-eryAIII gene encompassing the loading
didomain, module 1 and module 2 from DEBS1 and the
thioesterase from DEBS3. The strategy to obtain this
'diketide synthase' was to remove the DNA encoding part

44

of module 1, the whole of module 2 and part of the thioesterase, by digestion of plasmid pKW11 with EcoRV and EcoR1, and then to reconstitute module 1 and the N-terminal part of ACP1 by insertion of an appropriate PCR product, and similarly a PCR product was designed to replace the C-terminal part of ACP2 and the thioesterase. The two PCR products are joined by a unique BglII site created in the active site of the ACP, which involves an alteration in amino acid sequence of the hybrid ACP domain from EL (glutamic acid followed by leucine) as found in both ACP1 and ACP2 domains, to DL (aspartic acid followed by leucine). Such alterations in sequence at a PKS active site, with a view to retaining function, have not been previously attempted and it is not obvious that such altered sites should remain active.

Construction of plasmids pKW12, pKW13, pKW14 and pKW15

For the PCR amplification of DNA for module 1, the following synthetic oligonucleotides were used as mutagenic primers, one containing an EcoRV site and the other a BglII site:
5'-GCAGGGATATCGCACGTTCCTGG-3'
and 5'-CGCCGAGATCTGCGAAGGCCTGGTCGGCGGG-3'

PCR was carried out on pNTEP2 as template using Pfu DNA polymerase and 30 cycles of 95% (1 min); annealing at 55%C (1 min) and extension at 72%C (2 min), in the presence of 10% (vol/vol) dimethylsulphoxide. The product (PCR1) was end-repaired and cloned into SmaI-cut phagemid pUC119 and the ligation mixture was used to transform E. coli TG1recO. Plasmid DNA was prepared from individual colonies and the desired plasmid (5.0 kbp) was identified by its restriction pattern and was designated pKW12.

For PCR amplification of the DNA for the 5' end of module

2 and the thioesterase domain, the following
oligonucleotides containing respectively a Bgl II site
and an EcoRI site, were used as mutagenic primers:

5'-ATGAATTCCCTCCGCCCAGCCAG-3'
and
5'-ACAGATCTCGGCTTCGACTCGCTGACCG-3'

PCR was carried out on pNTEP2 as template exactly as
described above for PCR1 and the product (PCR2) was end-
repaired and cloned into SmaI-cut phagemid pUC119. The
ligation mixture was used to transform E. coli TG1recO
and plasmid DNA was prepared from individual colonies.
The desired plasmid (4.1 kbp) was identified by its
restriction pattern and was designated pKW13.

Plasmid pKW12 was digested with EcoRV and HindIII, and
the 1.8 kbp insert was end-repaired, and then ligated
together with plasmid pKW11 which had been linearised
with EcoRV and treated with alkaline phosphatase. The
ligation mixture was transformed into E. coli TG1recO and
the plasmid content of individual colonies was checked.
The desired plasmid (15.8 kbp) was identified in which
the unique Eco RV site had been reconstituted, and this
plasmid was designated pKW14.

Plasmid pKW13 was digested with BglII and EcoRI and the
0.9 kbp insert was ligated into plasmid pKW14 which had
been digested with BglII and EcoRI. The ligation mixture
was transformed into E. coli TG1recO and the plasmid
content of the individual colonies was checked. The
desired plasmid (9.32 kbp) was identified, in which the
0.9 kbp BglII-EcoRI fragment of pKW13 replaced the 9.5
kbp BglII-EcoRI segment of pKW14, and this plasmid was
designated pKW15.

Example 21

46

Construction and use of plasmid pKW16

(i) Construction

Plasmid pKW15 was digested with NdeI and XbaI and the
insert was ligated into plasmid pRM52 which had also been
digested with NdeI and XbaI. The ligation was
transformed into E. coli TGI recO and isolated colonies
were screened for their plasmid content. The desired
plasmid was identified by its restriction map and was
designated pKW16.

(ii) Use of plasmid pKW16 for construction of S.
coelicolor CH999/pKW16

Plasmid pKW16 was used to transform the methylation-
deficient strain E. coli ET12567 (MacNeil, D. J. et al.
Gene (1992) 111:61-68) and the demethylated plasmid pKW16
DNA isolated from this strain was used to transform S.
coelicolor CH999 (McDaniel, R. et al. Science (1993)
262:1546-1550. S. coelicolor protoplasts were
transformed with pKW16 and stable thiostrepton resistant
colonies were transferred to tap water medium agar plates
containing 50 μg/ml thiostrepton.

(iii) Isolation and characterisation of (2S)-methyl-(3R)-
hydroxypentanoic acid and (2S)-methyl-(3R)-
hydroxybutanoic acid.

A colony of S. coelicolor CH999/pKW16 was picked and
transferred to 100 ml YEME supplemented with 50 μg/ml
thiostrepton and allowed to grow at 30°C. After 4 days
the broth was filtered to remove mycelia, acidified to pH
3.0 and solid sodium chloride added until the solution
was saturated. The broth was extracted 5 times with an
equal volume of ethyl acetate, and the combined ethyl
acetate extracts were dried by extraction with saturated

SUBSTITUTE SHEET (RULE 26)

47

sodium chloride solution and concentrated by evaporation.
Thin layer chromatography on silica gel plates, eluted
with ethyl acetate:acetic acid 99:1 (v/v) and stained
with potassium permanganate, showed the presence of a
compound with the same mobility (Rf 0.55) as a reference
sample of (2S)-methyl-(3R)-butanoic acid, which was not
present in an extract obtained from S. coelicolor CH999
alone. Electrospray mass spectrometry (ESMS) analysis,
in the negative ion mode, of the ethyl acetate extracts
showed a major peak at m/e 117 not present in the control
sample. In positive ion mode, and in the presence of
formic acid, a peak was observed at m/e 119, which
shifted to m/e 141 in the presence of added sodium ions.
The exact mass of the sodium adduct was determined to be
141.05171 (the sodium salt of 2-methyl-3-hydroxybutanoic
acid requires 141.05248). When a colony of S. coelicolor
CH999/pKW16 was picked and transferred to 100 ml YEME
supplemented with 50 μg/ml thiostrepton and allowed to
grow at 30°C for 7 days, an ethyl acetate extract
prepared as above showed an additional peak, in ESMS
operated in negative ion mode, at m/e 131. In ESMS
operated in positive ion mode, and in the presence of
added formic acid, the peak is found at m/e 155. The
exact mass of this peak was determined to be 155.06973
(the sodium salt of 2-methyl-3-hydroxypentanoic acid
requires 155.06890).

Example 22
Construction of plasmid pAR33

Plasmid pAR33 contains a hybrid Type I PKS comprising the
ery loading module, extension module 1 of the ery PKS,
extension module 12 of the rap PKS, and the ery chain-
terminating thioesterase. It is constructed via several
intermediate plasmids as follows (Figure 13):

Construction of plasmid pARRAP

48

The 4.7 kbp DNA segment of the rapC gene encoding module
12 of the rapamycin PKS was amplified by PCR employing
the CloneAmp procedure (Raschtian, A. et al. Anal.
Biochem. (1992) 91:91-97) and with the following two
oligonucleotides as primers:
5'-
ACGCGUACUAGUCAGATCTGGGCATCAATTCGCTGACCGCGGTGGAACTGCGCAA-
3'
and 5'-
AUGGAGAUCUCUCAGATCTTGAATGCGGCGGCTGCGGGGATGGTGCTGGCGTCA-
3', and using as template the DNA of clone_λ-1C
(Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995)
92:7839-7843). Approximately 30-60 ng of the PCR product
(4.7 kbp) is digested with uracil DNA glycosylase for 30
minutes at 37°C in the presence of 25 ng pAMP18 vector
DNA (Gibco BRL), the mixture is cooled on ice and
transformed into E. coli TG1recO and individual colonies
are checked for their plasmid content. The desired
plasmid (7.4 kbp) is identified by its restriction map
and is designated pARRAP.

Construction of plasmid pAR32

Plasmid pARRAP is digested with BglII to release the 4.7
kbp fragment encoding rap module 12, which is purified by
gel electrophoresis and then ligated into plasmid pKW15,
which has been linearised by digestion with BglII. The
ligation mixture is transformed into E. coli TG1recO and
individual colonies are checked for their plasmid
content. The desired plasmid is one in which the rap
module 12 has the correct orientation with respect to the
coding sequence of the open reading frame of the insert
in pKW15, so that a hybrid triketide lactone synthase
gene is produced. Such a plasmid is identified by its
restriction pattern, and is designated pAR32.

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49

Construction of plasmid pAR33

Plasmid pAR32 contains an insert that can be excised by digestion with NdeI and XbaI, but there is an additional NdeI site in the insert that must be specifically protected against cleavage. This is done using the RecA protection method (Koob, M. et al. Nucl. Acids Res. (1992) 20:5831-5835)). The synthetic oligonucleotide 5'-GCACCCACGACGCCACCACCACATATGCCCTGCACCCTGCCCTCC-3' (in which the NdeI site is underlined) is used together with purified RecA protein and ATP_S, to form a stable triplex DNA-protein complex that specifically protects the internal NdeI site in rap module 12 from digestion. The protected plasmid pAR32 is digested with NdeI and XbaI, producing the desired full-length insert (13.1 kbp), and this is ligated with plasmid pRM52 (Example 4) which has been digested with NdeI and XbaI. The ligation mixture is transformed into E. coli TG1 recO and individual colonies are screened for their plasmid content. The desired plasmid pAR33 is identified by its restriction pattern.

Example 23
Construction of S. erythraea JC2/pAR33 and preparation of TKL derivatives

(i) Construction

Approximately 5 μg of plasmid pAR33 is transformed into protoplasts of S. erythraea JC2 and stable thiostrepton resistant colonies are selected. Total DNA from one such colony is isolated and analysed by Southern hybridisation, to confirm that the plasmid has integrated specifically into the chromosomal copy of the portion of the eryAIII gene that encodes the C-terminal thioesterase/cyclase. This strain is designated S. erythraea JC2/pAR33.

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50

(ii) Production of a novel triketide lactone by S.
erythraea JC2/pAR33

S. erythraea JC2/pAR33 is inoculated into sucrose-
succinate medium containing 50 μg/ml thiostrepton, and
allowed to grow for five days at 28-30%C. After this
time, the broth is filtered, and extracted twice with an
equal volume of ethyl acetate, and the combined ethyl
acetate extracts are dried over anhydrous sodium sulphate
and the ethyl acetate is removed by evaporation under
reduced pressure. Electrospray MS of the residue showed
the presence of Ac-2-nor-3-epi-TKL (III, R=Me) and 2-nor-
3-epi-TKL (III, R=Et).

Example 24
Construction of plasmid pAR8

Construction of a hybrid triketide lactone synthase
containing the ery loading didomain and ery chain-
terminating thioesterase/cyclase, and modules 11 and 12
of the rap PKS

This example requires the initial construction of five
separate plasmids, four housing separate elements of the
target construct, and a fifth housing a gene conferring
resistance to tetracycline. The inserts in these
plasmids are sequentially combined by standard in vitro
recombinant DNA techniques to form plasmid pAR5. A
further three cloning steps lead to the final expression
plasmid pAR8 (Figure 14).

Construction of plasmid pARLD

The segment of the ery AI gene from nucleotide 1 to
nucleotide 1673, encoding the loading AT-ACP didomain,
was amplified by PCR employing the CloneAmp procedure

51

with the following two oligodeoxynucleotides as primers:

5'-
ACGCGUACUAGUCCGATTAATTAAGGAGGACCATCAATGGCGGACCTGTCAAAGCT
C-3' and
5'-
AUGGAGAUCUCUCCGCTAGCGGTTCGCCGGGCGCCGCTTCGTTGGTCCGCGCGCGGG
TTTCCC-3'

and plasmid pBK6.12 (Example 5) as template, to give
plasmid pARLD.

Construction of plasmid pAR11

The segment of the rapC gene of S. hygroscopicus
(Schwecke, T. et al. Proc. Natl. Acad. Sci. USA (1995)
92:7839-7843) from nucleotide 112 to nucleotide 2095, the
5'- end of the DNA encoding rap module 11, is amplified
by PCR employing the CloneAmp procedure with the
following two oligodeoxynucleotides as primers:

5'-AUGGAGAUCUCUCCGCTAGCGATTGTGGGTATGGCG-3'
and
5'-ACGCGUACUAGUCCATGCATCTGCAGCACGGCGGCCTCATCACCGGA-3'
and the DNA of recombinant bacteriophage _λ-1C (Schwecke,
T. et al., Proc. Natl. Acad. Sci. USA (1995) 92:7839-
7843) as the template. Approximately 30-60 ng of the PCR
product (2.0 kbp) is digested with uracil DNA glycosylase
for 30 min at 37%C in the presence of 25 ng pAMP18 vector
DNA, the mixture is cooled on ice and transformed into E.
coli TG1 recO and individual colonies checked for their
plasmid content. The desired plasmid (4.7 kbp) is
identified by its restriction map and is designated
pAR11.·

Construction of plasmid pAR12

52

The segment of the rapC gene of S. hygroscopicus
(Schwecke, T. et al., Proc. Natl. Acad. Sci. USA (1995)
92:7839-7843) from nucleotide 7405 to nucleotide 9396,
the 3' end of the DNA encoding rap module 12, is
amplified by PCR employing the CloneAmp procedure with
the following two oligodeoxynucleotides as primers:

5'-ACGCGUACUAGUCCATGCATTCCCGGAGCGGCGATCTGTGG-3'
and
5'-AUGGAGAUCUCUCCCGCGGCCGCGCTGTCACGCACCAGCTTC
AGCAGTGCGTC-3' and the DNA of recombinant bacteriophage
λ-1C (Schwecke, T. et al., Proc. Natl. Acad. Sci. USA
(1995) 92:7839-7843) as template. Approximately 30-60 ng
of the PCR product (2.0 kbp) is digested with uracil DNA
glycosylase for 30 minutes at 37%C in the presence of 25
ng pAMP18 vector DNA, the mixture is cooled on ice and
tranformed into E. coli TG1recO and individual colonies
are checked for their plasmid content. The desired
plasmid (4.7 kbp) is identified by its restriction map
and is designated pAR12.

Construction of pARTE

The 1.3 kbp segment of the eryAIII gene, extending by 132
nucleotides 3' of the eryAIII stop codon to a KpnI site,
and encoding the C-terminal chain-terminating
thioesterase/cyclase of DEBS, is amplified by PCR
employing the CloneAmp procedure with the following two
oligodeoxynucleotides as primers:
5'-ACGCGUACUAGUCCGCGGCCGCGATCCTCGGGCATTCCAGC-3'
and
5'-AUGGAGAUCUCUAAGCATTGGTAACTGTC-3', and plasmid pEXDB3
(Roberts, G. A. et al. Eur J. Biochem. (1993) 214:305-
311) as the template. Approximately 30-60 ng of the PCR
product (1.3 kbp) is digested with uracil DNA glycosylase
for 30 min at 37%C in the presence of 25 ng pAMP18 vector
DNA, the mixture is cooled on ice and transformed into E.

53

coli TG1 recO and individual colonies checked for their
plasmid content. The desired plasmid (4.0 kbp) is
identified by its restriction map and is designated
pARTE.

Construction of plasmid pARTr

The 1.3 kbp segment of plasmid pBR322 containing the
tetracycline resistance gene is amplified by the CloneAmp
procedure with the following two oligodeoxynucleotides as
primers:

5'-ACGCGUACUAGUATCTAGACCATGCATGTTTGACAGCTTATCATC-3'
and
5'-AUGGAGAUCUCUATCTAGACCATGCATGCCGCCGGCTTCCATTCA-3'

and plasmid pBR322 as the template. Approximately 30-60
ng of the PCR product (1.3 kbp) is digested with uracil
DNA glycosylase for 30 minutes at 37%C in the presence of
25 ng pAMP18 vector DNA, the mixture is cooled on ice and
transformed into E. coli TG1recO and individual colonies
are checked for their plasmid content. The desired
plasmid (4.0 kbp) is identified by its restriction map
and is designated pARTr.

Construction of plasmid pAR1

Plasmid pARLD is digested with NheI and HindIII, and
ligated to the 2.0 kbp NheI-HindIII insert obtained from
plasmid pAR11. The ligation mixture is transformed into
E. coli TG1recO and individual colonies are checked for
their plasmid content. The desired plasmid is identified
by its restriction map and is designated pAR1.

Construction of plasmid pAR2

Plasmid pAR1 is linearised with NsiI and ligated with the

54

NsiI fragment from pARTr. The ligation mixture is
transformed into E. coli TG1recO and individual colonies
are checked for their plasmid content. The desired
plasmid is identified by its restriction map and is
designated pAR2.

Construction of plasmid pAR3

Plasmid pAR2 is digested with SpeI and XbaI and the
insert is ligated with plasmid pAR12 which has been
linearised with SpeI. The ligation mixture is
transformed into E. coli TG1recO and individual colonies
are checked for their plasmid content. The desired
plasmid is identified by its restriction map and is
designated pAR3.

Construction of plasmid pAR4

Plasmid pAR3 is digested with NsiI and the vector is
ligated to the NsiI fragment of pARTr, containing the
tetracycline resistance gene. The ligation mixture is
transformed into E. coli TG1recO and individual
colonies, grown in the presence of tetracycline (12.5
μg/ml), are checked for their plasmid content. The
desired plasmid is identified by its restriction map and
is designated pAR4.

Construction of plasmid pAR5

Plasmid pAR4 is digested with NotI and XbaI and ligated
with a NotI-XbaI fragment obtained by digestion of
plasmid pARTE. The ligation mixture is transformed into
E. coli TG1recO and individual colonies, grown in the
presence of tetracycline (12.5 μg/ml), are checked for
their plasmid content. The desired plasmid is identified
by its restriction map and is designated pAR5.

55

Construction of plasmid pAR5-2

A 7.2 kbp segment of the rapC gene of S. hygroscopicus is
excised from cosmid 13 (Schwecke, T. et al., Proc. Natl.
Acad. Sci. USA (1995) 92:7839-7843) using BstXI and NdeI,
purified by gel electrophoresis, and ligated with plasmid
pAR5 which has also been digested with BstXI and NdeI.
The ligation mixture is transformed into E. coli TG1recO
and individual colonies, grown in the presence of
tetracycline (12.5 µg/ml), are checked for their plasmid
content. The desired plasmid (11.9 kbp) is identified by
its restriction map and is designated pAR5-2.

Construction of plasmid pAR5-3

A 3.0 kbp segment of plasmid pAR5 is excised by digestion
with NdeI, purified by gel electrophoresis, and ligated
with plasmid pAR5-2 which had been linearised with NdeI.
The ligation mixture is transformed into E. coli TG1recO
and individual colonies, grown in the presence of
tetracycline (12.5 µg/ml), are checked for their plasmid
content. The desired plasmid (14.9 kbp) is identified by
its restriction map and is designated pAR5-3.

Construction of plasmid pAR8

A 12.2 kbp fragment of plasmid pAR5-3 is excised using
PacI and XbaI, purified by gel electrophoresis, and
ligated with plasmid pRM52 (Example 4) which had been cut
with PacI and XbaI. The ligation mixture is transformed
into E. coli TG1recO and and individual colonies, grown
in the presence of tetracycline (30.3 µg/ml), are checked
for their plasmid content. The desired plasmid (14.9
kbp) is identified by its restriction map and is
designated pAR8.

Example 25

56

Construction of S. erythraea JC2/pAR8 and production of
TKL derivatives

(i) Construction

Approximately 5-10 μg pAR8, isolated from E. coli DH10B
(pAR8) is used to transform S. erythraea JC2 protoplasts
and stable thiostrepton resistant colonies are selected.
One of these colonies is selected and total DNA is
prepared for Southern hybridisation analysis, to confirm
that the plasmid has integrated specifically into the
chromosomal copy of the portion of the eryAIII gene that
encodes the C-terminal thioesterase/cyclase. This strain
is designated S. erythraea JC2/pAR8

(ii) Production of 2,4-bisnor-3-epi-TKL and (Ac)-2,4-
bisnor-3-epi-TKL

A colony of S. erythraea JC2/pAR8 is picked and
transferred to sucrose-succinate medium supplemented with
50 μg/ml thiostrepton and allowed to grow at 30°C. After
3 days the broth is filtered and extracted twice with an
equal volume of ethyl acetate. The combined ethyl
acetate extracts are dried over anhydrous sodium sulphate
and concentrated under reduced pressure. GC-MS of the
residue shows the presence of 2,4-bisnor-3-epi-TKL
(IV,R=E+) and (Ac)-2,4-bisnor-3-epi-TKL (IV, R=Me)

Example 26
Construction of plasmid pE1A2TE

Plasmid pE1A2TE (like plasmid pE1A2TE-2 also described
herein) consists of a pT7.7 derived plasmid containing a
hybrid Type I PKS gene comprising the ery loading module,
the first extension module of the ery PKS, then the
second extension module of the avr PKS, and the

57

thioesterase of the ery PKS. It is constructed via
several intermediate plasmids as follows (Figure 15).

Construction of plasmid pIG70

Plasmid pVE1446 which contains a portion of the
avermectin PKS genes was obtained from E. coli ATCC
68250. Plasmid pVE1446 was digested with BamHI and the
7.0 kbp fragment between coordinates 6.05 and 13.05 was
purified by gel electrophoresis and ligated into plasmid
pUC119 which had been linearised with BamHI. The
ligation mixture was used to transform E. coli TG1 recO
and individual colonies were checked for their plasmid
content. Of the two possible orientations of the BamHI
insert pIG70 was selected such that when digested with
PstI fragments of approximately 2.0 and 8.6 kbp were
obtained and when digested with EcoRI fragments of
approximately 5.1 and 5.5 kbp were obtained.

Construction of plasmid pIG71

Plasmid pVE1446 which contains a portion of the
avermectin PKS genes was obtained from E. coli ATCC
68250. Plasmid pVE1446 was digested with BamHI and the
7.1 kbp fragment between coordinates 13.05 and 20.15 was
purified by gel electrophoresis and ligated into plasmid
pUC119 which had been linearised with BamHI. The
ligation mixture was used to transform E. coli TG1 recO
and individual colonies were checked for their plasmid
content. Of the two possible orientations of the BamHI
insert pIG71 was selected such that when digested with
EcoRI and XhoI 2 fragments of approximately 5 kbp were
obtained.

Construction of plasmid pIG70ΔPst

pIG70 was cut with Pst1 and religated. pIG70ΔPst was

58

isolated after transformation into E. coli TG1 recO.

Construction of plasmid pIG70ΔEco

pIG70 was cut with EcoRI and religated. pIG70ΔEco was
isolated after transformation into E. coli TG1 recO

Construction of plasmid pIG71ΔSac

pIG71 was cut with SacI and religated. pIG71ΔSac was
isolated after transformation into E. coli TG1 recO

Construction of plasmid pIGPCRstart

50 pmol of each of synthetic oligonucleotides 8985 (5'-
GAGCAGTCGTTCCGAGATCTCGGCTTCGATTCA-3') which introduced a
BglII site and 9204 (5'-GGGAGGAGATCAGATCCCAGAAGT-3') were
used by PCR to amplify a 300 bp product from 60 ng
pIG70ΔEco. The PCR product was end-repaired,
phosphorylated and ligated into pUC18 that had been
linearised with SmaI and dephosphorylated. The ligation
mixture was used to transform E. coli TG1 recO and
individual colonies were checked for their plasmid
content. The orientation of pIGPCRstart was identified
by a double restriction enzyme digest with EcoRI and
BglII to give a pattern that included a 300 bp fragment.

Construction of plasmid pIGPCRend

50 pmol of each of synthetic oligonucleotides 8986 (5'-
GAGGGAGTCGAACCGAGATCTCGGAACGCGCGG-3') which introduced a
BglII site and 9205 (5'-GGGGGATCCTGGGGTCGGCCGGGCAGGGCAA-
3') were used by PCR to amplify a 440 bp product from 60
ng pIG71ΔSac. The PCR product was end-repaired,
phosphorylated and ligated into pUC18 that had been
linearised with SmaI and dephosphorylated. The ligation
mixture was used to transform E. coli TG1 recO and

individual colonies were checked for their plasmid
content. The orientation of pIGPCRend was identified by
its restriction enzyme digest pattern.

Construction of plasmid pIGstart+middle

Plasmid pIGPCRstart was digested with PstI and the 300 bp
fragment was purified by gel electrophoresis and ligated
into plasmid pIG70ΔPst which had been linearised with
PstI and dephosphorylated. The ligation mixture was used
to transform E. coli TG1 recO and individual colonies
were checked for their plasmid content. Plasmids which
contained the correct orientation of the PstI-PstI insert
were identified by DNA sequencing.

Construction of plasmid pIGAve2Bgl

Plasmid pIGstart+middle was digested with BamHI and the
5.0 kbp fragment was purified by gel electrophoresis and
ligated into plasmid pIGPCRend which had been cut with
BamHI and dephosphorylated. The ligation mixture was
used to transform E. coli TG1 recO and individual
colonies were checked for their plasmid content.
Plasmids which contained the correct orientation of the
BamHI-BamHI insert were identified by DNA sequencing.

Construction of plasmid pE1A2TE

Plasmid pIGAve2Bgl was digested with BglII and the 6 kbp
fragment was purified by gel electrophoresis and ligated
into plasmid pKW15 (Example 16) which had been linearised
with BglII and dephosphorylated. The ligation mixture
was used to transform E. coli TG1 recO and individual
colonies were checked for their plasmid content.
Plasmids which contained the correct orientation of the
BglII-BglII insert were identified by restriction enzyme
digest with EcoRI.

60

Example 27
Construction and use of plasmid pIG2

(i) Construction
Plasmid pE1A2TE was digested with NdeI and XbaI and the
11 kbp fragment was purified by gel electrophoresis and
ligated into plasmid pRM52 (Example 4) which had been cut
with NdeI and XbaI. The ligation mixture was used to
transform E. coli TG1 recO and individual colonies were
checked for their plasmid content.

(ii) Construction of S. coelicolor CH999/pIG2

Plasmid pIG2 which had been isolated from E. coli ET12567
(MacNeil, D. J. et al. Gene (1992) 111:61-68) was
transformed into protoplasts of S. coelicolor CH999 and
stable thiostrepton resistant colonies were isolated.
Individual colonies were checked for their plasmid
content and the presence of plasmid pIG2 was confirmed by
its restriction pattern.

Example 28
Construction of plasmid pIG102

Plasmid pE1A2TE was digested with NdeI and XbaI and the
11 kbp fragment was purified by gel electrophoresis and
ligated into plasmid pCJR101 (Example 2) which had been
cut with NdeI and XbaI. The ligation mixture was used to
transform E. coli TG1 recO and individual colonies were
checked for their plasmid content.

Example 29

(i)Construction of plasmid pKS22

Plasmid pKS22 is a pNTEP2-derived vector containing a

61

DEBS1 -TE-derived triketide synthase with a KS2 domain in
the place of the KS1 domain. Plasmid pKS22 is obtained
via several intermediate plasmids as follows (Figure16).

Construction of plasmids pMO07, pMO08 and pMO09

For the PCR amplification for plasmid pMO07, the
following synthetic oligonucleotides were used as
mutagenic primers, one containing a HindIII site and
the other an EcoRV site:
5' -GTCTCAAGCTTCGGCATCAGCGGCACCAA- 3'
and 5' -CGTGCGATATCCCTGCTCGGCGAGCGCA-3'

For the PCR amplification for plasmid pMO08, the
following synthetic oligonucleotides were used as
mutagenic primers, one containing a PstI site and
the other a HindIII site:
5' -CATGGCCTGCAGGCTGCCCGGGGAGGTCGACT- 3'
and 5' -CCCGAAGCTTGACACACCTGCCCGGCGCACCCCGT- 3'

For the PCR amplification for plasmid pMO09, the
following synthetic oligonucleotides were used as
mutagenic primers, one containing a MunI site and
the other a PstI site:
5' -GCGCGCCAATTGCGTGCACATCTCGAT- 3'
and 5' -CCTGCAGGCCATCGCGACGACCGCGACCGGTTCGCCG- 3'

PCR was carried out on pNTEP2 as template using Pwo
DNA polymerase and one cycle of: 96°C (1min);
annealing at 50° C (3min); and extension at 72°C
(1min), and 25 cycles of: 96°C (1min); annealing at
50°C (1min); and extension at 72°C (1min) in the
presence of 10% (vol/vol) dimethylsulphoxide. The
products were end-repaired and cloned into pUC18
digested with SmaI and the ligation mixture was
transformed into E. coli DH 10B. Plasmid DNA was
prepared from individual colonies. The desired

62

plasmids for pMO07 (3.8kbp), pMO08 (3.9 kbp) and
pMO09 (4.3 kbp) were identified by their restriction
pattern and DNA sequencing.

Plasmid pMO08 was digested with HindIII, and the
1.2 kbp insert was cloned into pMO07 which had been
digested with HindIII. The ligation mixture was
transformed into E. coli DH 10B. The desired
plasmid (5.0 kbp) was identified by its restriction
pattern and designated pMO10.

Plasmid pMO09 was digested with PstI, and the 1.6
kbp insert was cloned into pMO10 which had been
digested with PstI. The ligation mixture was
transformed into E. coli DH 10B. The desired
plasmid (6.6 kbp) was identified by its restriction
pattern and designated pMO11.

Plasmid pMO11 was digested with MunI and EcoRV, and
the 3.9 kbp fragment was cloned into pNTEPH (see
below) which had been digested with MunI and EcoRV.
The ligation mixture was transformed into E. coli DH
10B. The desired plasmid (13 kbp) was identified by
its restriction pattern and designated pKS22.

Plasmid pNTEPH was obteined from pNTEP2 by removing
the HindIII site. pNTEP2 was digested with HindIII,
the 5' overhang was filled in with Klenow Fragment
DNA Polymerase I and religated. The desired plasmid
(13.6 kbp) was identified by its restriction
pattern.

Example 30

(i).Construction of plasmid pIB018

Plasmid pIB018 is a pCJR24-derived vector containing

a DEBS1TE-derived triketide synthase with KS1 in the
place of KS2. Plasmid pIB018 is obtained via several
intermediate plasmids as follows (Figure17A).

Construction of plasmids pKSA, pKSB and pKSC

For the PCR amplification for plasmid pKSA, the
following synthetic oligonucleotides were used as
mutagenic primers, one containing a PstI site and
the other a HindIII site:
5' -GATGGCCTGCAGGCTGCCCGGCGGTGTGAGCA- 3'
and 5' -GCCGAAGCTTGAGACCCCCGCCCGGCGCGGTCGC- 3'

For the PCR amplification for plasmid pKSB, the
following synthetic oligonucleotides were used as
mutagenic primers, one containing an EspI site and
the other a PstI site:
5' -TGGCTTCGCTGGCGGACACGCTCAG- 3'
and 5' -CCTGCAGGCCATGCCGACGATCGCGATCGGCT- 3'

For the PCR amplification for plasmid pKSC, the
following synthetic oligonucleotides were used as
mutagenic primers, one containing a HindIII site and
the other a BspEI site:
5' -GTCAAGCTTCGGGGTGAGCGGGACGAA- 3'
and 5' -GCGTCCGGACGTGGCTCCAGCA-3'

PCR was carried out on pNTEP2 as template using Pwo
DNA polymerase and one cycle of: 96° (1min);
annealing at 50° (3min); and extension at 72°
(1min), and 25 cycles of: 96°C (1min); annealing at
50°C (1min); and extension at 72°C (1min) in the
presence of 10% (vol/vol) dimethylsulphoxide. The
products were end-repaired and cloned into pUC18
digested with SmaI and the ligation mixture was
transformed into E. coli DH 10B. Plasmid DNA was
prepared from individual colonies. The desired

64

plasmids for pKSA (4.0 kbp), pKSB (4.2 kbp) and pKSC
(3.2 kbp) were identified by their restriction
pattern.

Plasmid pKSA was digested with PstI, and the 1.2 kbp
insert was cloned into pKSB which had been digested
with PstI. The ligation mixture was transformed
into E. coli DH 10B. The desired plasmid (5.5 kbp)
was identified by its restriction pattern and
designated pKSD.

Plasmid pKSC was digested with HindIII, and the 0.5
kbp insert was cloned into pKSC which had been
digested with HindIII. The ligation mixture was
transformed into E. coli DH 10B. The desired plasmid
(6.0 kbp) was identified by its restriction pattern
and designated pKSE.

Plasmid pKSE was digested with EspI and BspeEI, ant
the 3.3 kbp fragment was cloned into pUCTE which had
been digested with EspI and BspeEI. The ligation
mixture was transformed into E. coli DH 10B. The
desired plasmid (13.9 kbp) was identified by its
restriction pattern and designated pIB004.

Plasmid pIB004 was digested with NdeI and XbaI, and
the 11.2 kbp insert was cloned into pCJR24 which
had been digested with NdeI and XbaI. The ligation
mixture was transformed into E. coli DH 10B. The
desired plasmid (15.9 kbp) was identified by its
restriction pattern and designated pIB018.

(ii)Use of plasmid pIB018 for contruction of S.
erythraea.NRRL2338/pIB018

Approximately 5µg plasmid pIB018 is transformed into
protoplasts of S. erythraea NRRL 2338 and stable

65

thiostrepton resistant colonies are isolated. From
several colonies total DNA is obtained and analysed
by Southern hybridisation, to confirm that the
plasmid has integrated into the end of module2 of
eryAI.

S. erythraea NRRL2338/pIB018 is inoculated into
tryptic soy broth containing 50 μg/ml thiostrepton
and allowed to grow for three days at 30°C. 20 ml of
this seed culture are used to inoculate 400 ml of
sucrose-succinate medium containing 50 μg/ml
thiostrepton in a 2L flask with a single spring to
reduce clumping, shaken at 300 rpm. After 6 days the
broth was filtered, adjusted to pH 4 and extracted
three times with an equal volume of ethyl acetate.
The solvent was removed by evaporation. Triketide
lactone products (10 mg/L) were identified by GC-MS
and NMR. The major component was (2R, 3S, 4S, 5R)-
2,4-dimethyl-3,5-dihydroxy-n-hexanoic acid δ
lactone; (2R, 3S, 4S, 5R)-2,4-dimethyl-3,5-
dihydroxy-n-heptanoic acid δ lactone was also found:
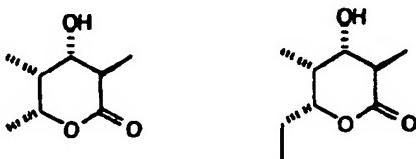
The following macrolides were identified by HPLC/MS:
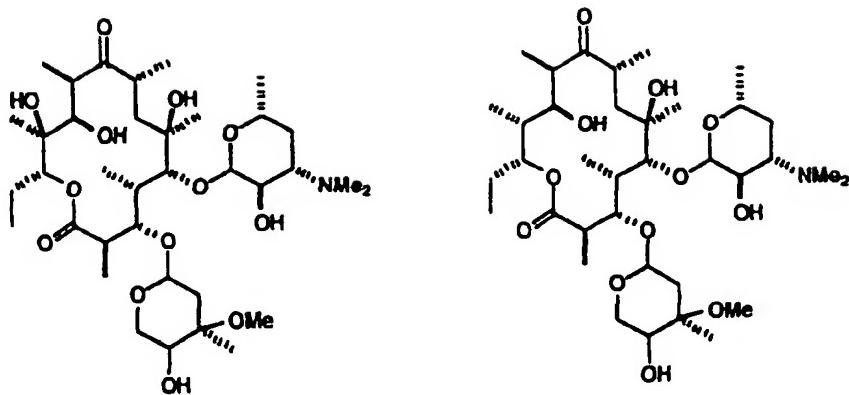
Example 31

(i) Construction of plasmid pIB017

Plasmid pIB017 is a pCJR24-derived vector containing
a DEBS1TE-derived triketide synthase with KS2 in the
place of KS1 and KS1 in the place of KS2. Plasmid
pIB017 is obtained via several intermediate plasmids
as follows (Figure 17B).

Plasmid pIB004 was digested with EcoRV and EcoRI,
and the 7.2 kbp fragment was cloned into pKS22 which
had been digested with EcoRV and EcoRI. The
ligation mixture was transformed into E. coli DH
10B. The desired plasmid (13.6kbp) was identified
by its restriction pattern and designated pIB009.

Plasmid pIB009 was digested with NdeI and XbaI, and
the 11.2 kbp insert was cloned into pCJR24 which had
been digested with NdeI and XbaI. The ligation
mixture was transformed into E. coli DH 10B. The

67

desired plasmid (15.9kbp) was identified by its
restriction pattern and designated pIB017.

(ii) Construction of S. erythraea NRRL2338/pIB017

Approximately 5μg plasmid pIB017 is transformed into
protoplasts of S. erythraea NRRL 2338 and stable
thiostrepton resistant colonies are isolated. From
several colonies total DNA is obtained and analysed
by Southern hybridisation, to confirm that the
plasmid has integrated into the end of module 2 of
eryAI.

S. erythraea NRRL2338/pIB017 is inoculated into
tryptic soy broth containing 50 μg/ml thiostrepton
and allowed to grow for three days at 30°C. 20 ml of
this seed culture are used to inoculate 400 ml of
sucrose-succinate medium containing 50 μg/ml
thiostrepton in a 2L flask with a single spring to
reduce clumping, shaken at 300 rpm. After 6 days
the broth was filtered, adjusted to pH 4 and
extracted three times with an equal volume of ethyl
acetate. The solvent was removed by evaporation.
Analysis of triketide lactones (0.4 mg/L) was done
by GC-MS, optical rotation and NMR. The compunds
isolated were found to be (2R, 3S, 4S, 5S)-2,4-
dimethyl-3,5-dihydroxy-n-hexanoic acid δ lactone;
and (2R, 3S, 4S, 5S)-2,4-dimethyl-3,5-dihydroxy-n-
heptanoic acid δ lactone.
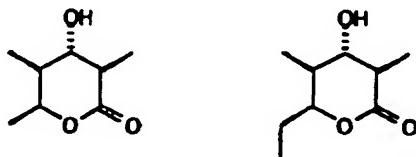
68

The following macrolides were identified by HPLC/MS:

Example 32

69
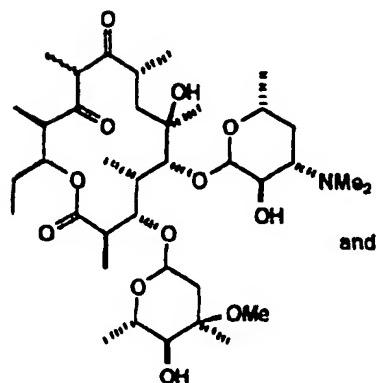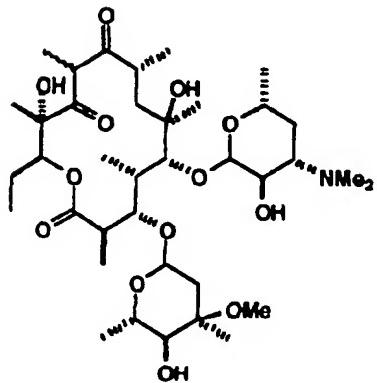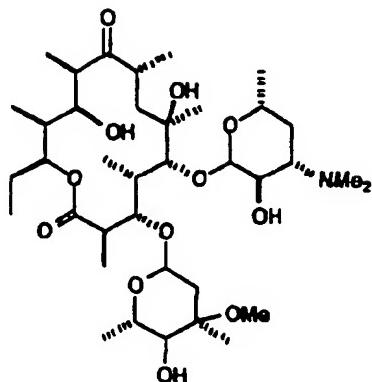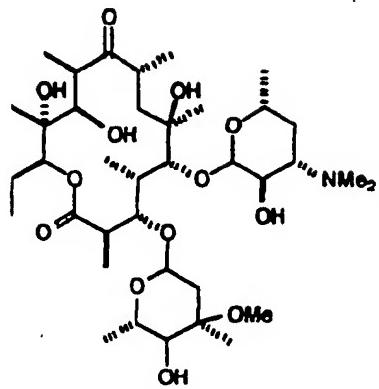
(I) Construction of plasmid pIB015

Plasmid pIB015 is a pCJR24-derived vector containing
a diketide synthase with LD, KS1, AT2, KR2, ACP2/6
and TE.   Plasmid pIB015 is obtained via several
intermediate plasmids as follows (Figure 18).

Plasmid pIB009 was digested with PstI to remove a
4.4 kbp fragment, and religated.   The ligation
mixture was transformed into E. coli DH 10B.   The
desired plasmid (9.2kbp) was identified by its
restriction pattern and designated pIB011.

Plasmid pIB011 was digested with NdeI and XbaI, and
the 6.8 kbp insert was cloned into pCJR24 which had
been digested with NdeI and XbaI.   The ligation
mixture was transformed into E. coli DH 10B.   The
desired plasmid (9.2 kbp) was identified by its
restriction pattern and designated pIB015.

(ii)Use of plasmid pIB015 for contruction of S.
erythraea JC2/pIB015

Approximately 5µg plasmid pIB015 is transformed into
protoplasts of S erythraea JC2 and stable
thiostrepton resistant colonies are isolated.   From
several colonies total DNA is obtained and analysed
by Southern hybridisation, to confirm that the
plasmid has integrated into the TE.

S. erythraea JC2/pIB015 is inoculated into tryptic
soy broth containing 50 µg/ml thiostrepton and
allowed to grow for three days at 30°C.   20 ml of
this seed culture are used to inoculate 400 ml of
sucrose-succinate medium containing 50 µg/ml
thiostrepton, 0.1 mg/ml 4-pentynoic acid and 0.1
mg/ml 3-tetradecylsulfanyl-propionic acid in a 2L

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flask with a single spring to reduce clumping,
shaken at 300 rpm. After 6 days the broth was
filtered, adjusted to pH 3 and extracted three times
with an equal volume of ethyl acetate. The solvent
was removed by evaporation. Analysis of diketide
acid was done by GC-MS equipped with a chiral column
(Hydrodex-β-PM 25 m x 0.25 mm ID (Machery-Nagel GmbH
& CoKG, Germany)) using all 4 synthetic
stereoisomers of the diketide acid as standards. The
compound produced was identified as (2R, 3S)-2-
methyl, 3-hydroxy pentanoic acid.

iii) Use of plasmid pIB015 for contruction of S.
erythraea ORF5/pIB015

Approximately 5μg plasmid pIB015 is transformed into
protoplasts of S. erythraea ORF5 and stable
thiostrepton resistant colonies are isolated. From
several colonies total DNA is obtained and analysed
by Southern hybridisation, to confirm that the
plasmid has integrated into module 2 of eryAI.

S. erythraea ORF5/pIB015 is inoculated into tryptic
soy broth containing 50 μg/ml thiostrepton and
allowed to grow for three days at 30°C. 20 ml of
this seed culture are used to inoculate 400 ml of
sucrose-succinate medium containing 50 μg/ml
thiostrepton in a 2L flask with a single spring to
reduce clumping, shaken at 300 rpm. After 6 days
the broth was filtered and extracted three times
with an equal volume of ethyl acetate. The solvent
was analysed by ESMS. The following compounds were
detected:

71
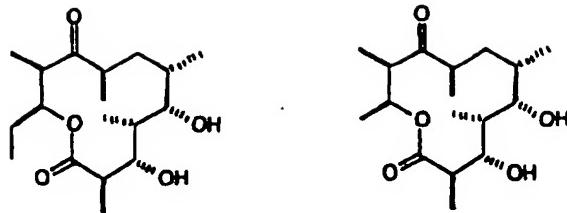
Example 33

(i)Construction of plasmid pIB016

Plasmid pIB016 is a pCJR24-derived vector containing
a diketide synthase with LD, KS2, AT2, KR2, ACP2/6
and TE. Plasmid pIB016 is obtained via several
intermediate plasmids as follows (Figure 18).

Plamid pIB009 was digested with HindIII to remove a
4.4 kbp fragment, and religated. The ligation
mixture was transformed into E. coli DH 10B. The
desired plasmid (9.2kbp) was identified by its
restriction pattern and designated pIB012.

Plamid pIB012 was digested with NdeI and XbaI, and
the 6.8 kbp insert was cloned into pCJR24 which had
been digested with NdeI and XbaI. The ligation
mixture was transformed into E. coli DH 10B. The
desired plasmid (9.2 kbp) was identified by its
restriction pattern and designated pIB016.

ii)Use of plasmid pIB016 for contruction of S.
erythraea ORF5/pIB016

Approximately 5μg plasmid pIB016 is transformed into
protoplasts of S. erythraea ORF5 and stable
thiostrepton resistant colonies are isolated. From

72

several colonies total DNA is obtained and analysed
by Southern hybridisation, to confirm that the
plasmid has integrated into module 2 of eryAI.

S. erythraea ORF5/pIB016 is inoculated into tryptic
soy broth containing 50 µg/ml thiostrepton and
allowed to grow for three days at 30°C. 20 ml of
this seed culture are used to inoculate 400 ml of
sucrose-succinate medium containing 50 µg/ml
thiostrepton in a 2L flask with a single spring to
reduce clumping, shaken at 300 rpm. After 6 days
the broth was filtered and extracted three times
with an equal volume of ethyl acetate. The extract
was analysed by ESMS. The following compounds were
detected:
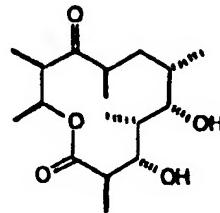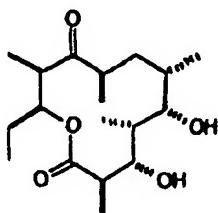
Example 34

Construction of plasmid pJLK15

Plasmid pJLK15 is a pCJR24 based plasmid containing a PKS
gene comprising the ery loading module, the first and the
second extension modules of the ery PKS and the ery
chain-terminating thioesterase except that the DNA
segment between the end of the acyltransferase and the
beginning of the ACP of the second ery extension module
has been substituted by the equivalent segment of module

13 of the rap PKS. It was constructed via several
intermediate plasmids as follows (Figure 19).

Construction of plasmid pJLK01

The approximately 0.46 kbp DNA fragment of the eryAI gene
of S. erythraea was amplified by PCR using as primers the
synthetic oligonucleotides: 5'-GGAGTACTGCGAGGGCGTGGGCAT-
3' and
5'-CACCTAGGACCGCTTCCCAGTCGACC-3' and plasmid pNTEPH as
template. The PCR product was treated with T4
polynucleotide kinase and then ligated with plasmid
pUC18, which had been linearised by digestion with SmaI
and then treated with alkaline phosphatase. The ligation
mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJLK01 was identified by its
restriction pattern and DNA sequencing.

Construction of plasmid pJLK08

The approximately 1.47 kbp DNA fragment of the eryAI gene
of S. erythraea was amplified by PCR using as primers the
synthetic oligonucleotides:
5'-TACCTAGGCCGGGCCGGACTGGTCGACCTGCCGGGTT-3' and
5'-ATCCTCAGGCTCTCCGTCTCCGGTTCTCC-3' and plasmid pNTEPH as
template. The PCR product was treated with T4
polynucleotide kinase and then ligated with plasmid
pUC18, which had been linearised by digestion with SmaI
and then treated with alkaline phosphatase. The ligation
mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJLK08 was identified by its
restriction pattern and DNA sequencing.

Construction of plasmid pJLK09

74

The approximately 1.12 kbp DNA fragment of the eryAI gene
of S. erythraea was amplified by PCR using as primers the
synthetic oligonucleotides:
5'-TACCTGAGGGACCGGCTAGCGGGTCTGCCGCGTG-3' and
5'-CTTCTAGACTATGAATTCCCTCCGCCCAGC-3' and plasmid pNTEPH
as template. The PCR product was treated with T4
polynucleotide kinase and then ligated with plasmid
pUC18, which had been linearised by digestion with SmaI
and then treated with alkaline phosphatase. The ligation
mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJLK09 was identified by its
restriction pattern and DNA sequencing.

Construction of plasmid pJLK10

Plasmid pJLK08 was digested with PstI and Bsu36I and the
insert was ligated with plasmid pJLK09 which had been
digested with PstI and Bsu36I. The ligation mixture was
used to transform E. coli DH10B and individual colonies
were checked for their plasmid content. The desired
plasmid pJLK10 was identified by its restriction pattern.

Construction of plasmid pJLK11

Plasmid pJLK01 was digested with PstI and AvrII and the
insert was ligated with plasmid pJLK10 which had been
digested with PstI and AvrII. The ligation mixture was
used to transform E. coli DH10B and individual colonies
were checked for their plasmid content. The desired
plasmid pJLK11 was identified by its restriction pattern.

Construction of plasmid pJLK12

Plasmid pJLK11 was digested with ScaI and the 4.7 kbp
fragment was ligated with plasmid pCJR34 which had been
digested with ScaI. The ligation mixture was used to

transform E. coli DH10B and individual colonies were
checked for their plasmid content. The desired plasmid
pJLK12 was identified by its restriction pattern.
pCJR34 was constructed in the following way. pNTEP2 was
digested with NdeI and XbaI and cloned into pUC19 which
had previously been digested with NdeI and XbaI. The
desired plasmid pCJR34 was identified by its restriction
pattern.

Construction of plasmid pJLK13

Plasmid pJLK12 was digested with NdeI and XbaI and the
11.2 kbp fragment was ligated with plasmid pCJR24 which
had been digested with NdeI and XbaI. The ligation
mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJLK13 was identified by its
restriction pattern.

Construction of plasmid pJLK14

The approximately 3.3 kbp DNA of the rapC gene of S.
hygroscopicus encoding the reduction loop of module 13
was amplified by PCR using as primers the synthetic
oligonucleotides:
5'-CGCCTAGGCACCACCACAACCCGGGTACTGGACC-3' and
5'-TAGCTAGCCGGGCGCTCAGGGGCTGCGAGCCGACCT-3' and cosmid cos
31 (Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci. USA
92:7839-7843) as template. The PCR product was treated
with T4 polynucleotide kinase and then ligated with
plasmid pUC18, which had been linearised by digestion
with SmaI and then treated with alkaline phosphatase. The
ligation mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJLK14 was identified by its
restriction pattern and DNA sequencing.

76

Construction of plasmid pJLK15

Plasmid pJLK14 was digested with AvrII and NheI and the
3.3 kbp fragment was ligated with plasmid pJLK13 which
had been digested with AvrII and NheI. The ligation
mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJLK15 was identified by its
restriction pattern.

Example 35

Use of plasmid pJLK15 for construction of JC2/pJLK15

Approximately 5 μg plasmid pJLK15 is transformed into
protoplasts of S. erythraea JC2 and stable thiostrepton
resistant colonies are isolated. From several colonies
total DNA is obtained and analysed by Southern
hybridisation, to confirm that the plasmid has integrated
into the TE. JC2/pJLK15 is inoculated into tryptic soy
broth containing 50 μg/ml thiostrepton and allowed to
grow for three days at 30°C. 20 ml of this seed culture
are used to inoculate 400 ml of sucrose-succinate medium
containing 50 μg/ml thiostrepton in a 2L flask with a
single spring to reduce clumping, shaken at 300 rpm.
After 6 days the broth was filtered, adjusted to pH 3 and
extracted three times with an equal volume of ethyl
acetate. The solvent was removed by evaporation and the
residue dissolved in methanol (5 ml) and analysed by
electrospray mass spectroscopy. The major products were
identified as (2R, 4R, 5R)-2,4-dimethyl-5-hydroxy-n-
hexanoic acid δ-lactone ($C_8H_{14}O_2$; MH$^+$: calc. 143.1072,
found 143.110; MNa$^+$: calc.165.0891, found 165.093) and as
(2R, 4R, 5R)-2,4-dimethyl-5-hydroxy-n-heptanoic acid δ -
lactone ($C_9H_{16}O_2$; MH$^+$: calc. 156.1150, found 156.118; MNa$^+$:
calc.178.0970, found 178.099).

77

Example 36

Construction of plasmid pJLK18

Plasmid pJLK18 is a pCJR24 based plasmid containing a PKS
gene comprising the ery loading module, the first and the
second extension modules of the ery PKS and the ery
chain-terminating thioesterase except that the DNA
segment between the end of the acyltransferase and the
beginning of the ACP of the second ery extension module
has been substituted by the equivalent segment of module
4 of the rap PKS. It was constructed via several
intermediate plasmids as follows (Figure 20).

Construction of plasmid pJLK16

The approximately 2.8 kbp DNA fragment of the rapA gene
of S. hygroscopicus encoding the reduction loop of module
4 was amplified by PCR using as primers the synthetic
oligonucleotides:
5'-CCTAGGCACCACCACGGCCCGGGTGCTGGACCTT -3' and
5'-CCTCAGGCTGTCACCGGTAGAGGCGGCCCT-3' and cosmid
cos 25 (Schwecke, T. et al. (1995) Proc. Natl. Acad. Sci.
USA 92:7839-7843) as template. The PCR product was
treated with T4 polynucleotide kinase and then ligated
with plasmid pUC18, which had been linearised by
digestion with SmaI and then treated with alkaline
phosphatase. The ligation mixture was used to transform
E. coli DH10B and individual colonies were checked for
their plasmid content. The desired plasmid pJLK16 was
identified by its restriction pattern and DNA sequencing.

Construction of plasmid pJLK17

Plasmid pJLK16 was digested with AvrII and Bsu36I and the
2.8 kbp fragment was ligated with plasmid pJLK12 which
had been digested with AvrII and Bsu36I. The ligation

mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJLK17 was identified by its
restriction pattern.

Construction of plasmid pJLK18

Plasmid pJLK17 was digested with NdeI and XbaI and the
11.2 kbp fragment was ligated with plasmid pCJR24 which
had been digested with NdeI and XbaI. The ligation
mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJLK18 was identified by its
restriction pattern.

Example 37

Use of plasmid pJLK18 for construction of JC2/pJLK18

Approximately 5 μg plasmid pJLK18 is used to transform
protoplasts of S. erythraea JC2 and stable thiostrepton
resistant colonies are isolated. From several colonies
total DNA is obtained and analysed by Southern
hybridisation, to confirm that the plasmid has integrated
into the TE. S. erythraea JC2/pJLK18 is inoculated into
tryptic soy broth containing 50 μg/ml thiostrepton and
allowed to grow for three days at 30°C. 20 ml of this
seed culture are used to inoculate 400 ml of sucrose-
succinate medium containing 50 μg/ml thiostrepton, 0.1
mg/ml 4-pentynoic acid and 0.1 mg/ml 3-
tetradecylsulfanyl-propionic acid in a 2L flask with a
single spring to reduce clumping, shaken at 300 rpm.
After 6 days the broth was filtered, adjusted to pH 3 and
extracted three times with an equal volume of ethyl
acetate. The solvent was removed by evaporation and the
residue dissolved in methanol (5 ml) and analysed by
electrospray mass spectroscopy. The major products were

identified as (E, 4R, 5R)-2,4-dimethyl-5-hydroxy-n-2-
hexenoic acid ($C_8H_{14}O_3$; MH$^+$: calc. 159.1021, found 159.098;
MNa$^+$: calc.181.0841, found 181.079) and (E, 4R, 5R)-2,4-
dimethyl-5-hydroxy-n-2-heptenoic acid ($C_9H_{16}O_2$; MH$^+$: calc.
173.1178, found 173.118; MNa$^+$: calc.195.0997, found
195.104).

Example 38

Construction of plasmid pJLK21 (Figure 21)

Construction of plasmid pJLK19

For the PCR amplification of an approximately 1.3
kbp DNA fragment for plasmid pJLK19, the following
synthetic oligonucleotides were used as primers:
5' -GTCAAGCTTCGGGGTGAGCGGGACGAA- 3'
and 5' -ATCCTAGGACCGCTTCCCAGTCGACCGCGACA- 3'
PCR was carried out on pNTEPH as template. The PCR
product was treated with T4 polynucleotide kinase
and then ligated with plasmid pUC18, which had
been linearised by digestion with SmaI and then
treated with alkaline phosphatase. The ligation
mixture was used to tranform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid pJKL19 was identified
by its restriction pattern.

Construction of plasmid pJLK20

Plasmid pIB011 was digested with HindIII and NdeI
and the 2.9 kbp fragment was cloned into pJKL19
which had been digested with HindIII and NdeI. The
ligation mixture was used to tranform E. coli
DH10B and individual colonies were checked for
their plasmid content. The desired plasmid pJKL20
was identified by its restriction pattern.

80

Construction of plasmid pJLK21

Plasmid pJKL20 was digested with AvrII and NdeI
cloned into pJLK15 which had been digested with
AvrII and NdeI. The ligation mixture was used to
tranform E. coli DH10B and individual colonies
were checked for their plasmid content. The
desired plasmid pJKL21 was identified by its
restriction pattern.

Example 39

Use of plasmid pJKL21 for construction of
JC2/pJKL21

Approximately 5 μg plasmid pJKL21 is transformed
into protoplasts of JC2 and stable thiostrepton
resistant colonies are isolated. From several
colonies total DNA is obtained and analysed by
Southern hybridisation, to confirm that the
plasmid has integrated into the thioesterase.
JC2/pJKL21 is inoculated into tryptic soy broth
containing 50 μg/ml thiostrepton and allowed to
grow for three days at 30 °C. 20 ml of this seed
culture are used to inoculate 400 ml of sucrose-
succinate medium containing 50 μg/ml thiostrepton,
0.1 mg/ml 4-pentynoic acid and 0.1 mg/ml 3-
tetradecylsulfanyl-propionic acid in a 2L flask
with a single spring to reduce clumping, shaken at
300 rpm. After 6 days the broth was filtered, the
pH adjusted to pH 3 and extracted 3 times with an
equal volume of ethyl acetate. The solvent was
removed by evaporation and the residue dissolved
in methanol (5 ml) and analysed by electrospray
mass spectroscopy. The major products were
identified as (2R)-2-methyl-butanoic acid ($C_5H_{10}O_2$;
MH$^+$: calc. 103.0759, found 103.071; MNa$^+$:

81

calc.125.0578, found 125.052) and as (2R)-2-
methyl-pentanoic acid.

Example 40

Construction of plasmid pJLK22

Plasmid pJKL20 was digested with AvrII and NdeI
cloned into pJLK18 which had been digested with
AvrII and NdeI. The ligation mixture was used to
tranform E. coli DH10B and individual colonies
were checked for their plasmid content. The
desired plasmid pJKL22 was identified by its
restriction pattern.

Example 41

Use of plasmid pJKL22 for construction of S.
erythraea JC2/pJKL22

Approximately 5 µg plasmid pJKL22 is transformed
into protoplasts of JC2 and stable thiostrepton
resistant colonies are isolated. From several
colonies total DNA is obtained and analysed by
Southern hybridisation, to confirm that the
plasmid has integrated into the thioesterase.

JC2/pJKL22 is inoculated into tryptic soy broth
containing 50 µg/ml thiostrepton and allowed to
grow for three days at 30 °C. 20 ml of this seed
culture are used to inoculate 400 ml of sucrose-
succinate medium containing 50 µg/ml thiostrepton,
0.1 mg/ml 4-pentynoic acid and 0.1 mg/ml 3-
tetradecylsulfanyl-propionic acid in a 2L flask
with a single spring to reduce clumping, shaken at
300 rpm. After 6 days the broth was filtered, the

pH adjusted to pH 3 and extracted 3 times with an
equal volume of ethyl acetate. The solvent was
removed by evaporation and the residue dissolved
in methanol (5 ml) and analysed by electrospray
mass spectroscopy. The major products were
identified as (E)-2-methyl-butenoic acid ($C_5H_8O_2$;
MH$^+$: calc. 101.0602, found 101.062; MNa$^+$:
calc.123.0422, found 123.043) and (E)-2-methyl-
pentenoic acid ($C_6H_{10}O_2$; MH$^+$: calc. 115.0759, found
115.077; MNa$^+$: calc.137.0578, found 137.058).

Example 42
For the construction of plasmid pKR1-0, a derivative of
pCJR24 which encodes a ketolactone synthase, several
intermediate plasmids were constructed (Figure 22).

Construction of plasmid p37

The 1.4 kbp segment of plasmid pNTEP2 containing from
nucleotide 9838 to 11214 (encoding amino acids 3279 to
the end of DEBS1-TE) is amplified by PCR with the
following two synthetic oligonucleotides as primers

5'-GCCACTAGTGTGGCGTGGGGGCTGTGGG-3' and
5'-TGAATTCCCTCCGCCCAGCCAGGCGTCGAT-3' and plasmid pNTEP2
as template. The PCR product was end-repaired and
ligated with plasmid pUC18, which had been linearised by
digestion with SmaI and then treated with alkaline
phosphatase. The ligation mixture was used to transform
E. coli DH10B and individual colonies were checked for
their plasmid content. The desired plasmid p37 in which
an SpeI site was introduced at the 5' end of this
fragment was identified by its restriction pattern and by
DNA sequencing.

Construction of plasmid p37N

83

Plasmid p37 was digested with EcoRI and KpnI and the 1.4 fragment was ligated to pNTEP2 previously digested with EcoRI and KpnI. The ligation mixture was used to transform E. coli DH10B and individual colonies were checked for their plasmid content. The desired plasmid p37N was identified by its restriction pattern.

Construction of plasmid pSCA7

The 1.1 kbp DNA segment of the eryAI gene of S. erythraea extending from nucleotide 8202 to nucleotide 9306 was amplified by PCR using as primers the synthetic oligonucleotides:
5'-CCTGGAGTACTGCGAGGGCGTG-3' and
5'-CTGACTAGTGGCGGTGACGTGGGCGGGGGAAA-3' and plasmid pNTEP2 as template. The PCR product was end-repaired and ligated with plasmid pUC18, which had been linearised by digestion with SmaI and then treated with alkaline phosphatase. The ligation mixture was used to transform E. coli DH10B and individual colonies were checked for their plasmid content. The desired plasmid pSCA7 in which an SpeI site has been introduced at the 3' end of this PCR product was identified by its restriction pattern and by DNA sequencing.

Construction of plasmid pSH

Plasmid p37N was digested with SpeI and HindIII and the 1.4 kbp fragment was ligated with plasmid pSCA7 previously digested with SpeI and HindIII. The ligation mixture was used to transform E. coli DH10B and individual colonies were checked for their plasmid content. The desired plasmid pSH was identified by its restriction pattern.

Construction of plasmid pUCTE

84

Plasmid pNTEP2 was digested with BglII and HindIII and
the 11.2 kbp insert was ligated to BamHI and HindIII
digested plasmid pUC18. The ligation mixture was used to
transform E. coli DH10B and individual colonies were
checked for their plasmid content. The desired plasmid
pUCTE was identified by its restriction pattern.

Construction of plasmid pUC1-0

The 3.9 kbp ScaI restriction fragment of pUCTE was
substituted for the 3.4 kbp ScaI restriction fragment of
pSH. The desired plasmid pUC1-0 was identified by its
restriction pattern.

Construction of plasmid pKR1-0

The 10.7 kbp NdeI and XbaI restriction fragment of pUC1-0
was ligated to NdeI and XbaI digested pCJR24. The
ligation mixture was used to transform E. coli DH10B and
individual colonies were checked for thier plasmid
content. The desired plasmid pKR1-0 was identified by
its restriction pattern.

Example   43

Construction and use of S. erythraea JC2/pKR1-0

(i) Construction
Approximately 5 μg of plasmid pKR1-0 was used to
transform protoplasts of S. erythraea JC2 and stable
thiostrepton resistant colonies were isolated. From
several such colonies, total DNA was obtained and
analysed by Southern hybridisation, to confirm that the
plasmid had integrated specifically into the portion of
the eryAIII gene that encodes the C-terminal
thioesterase/cyclase, by homologous recombination. One
such clone was selected and designated S. erythraea

85

JC2/pKR1-0.

(ii) Production of triketide lactones using S. erythraea
JC2/pKR1-0

S. erythraea JC2/pKR1-0 was inoculated into sucrose-
succinate medium containing 10 µg/ml thiostrepton and
allowed to grow for four days at 30°C. After this time
the broth is filtered to remove mycelia and then
extracted twice with ethyl acetate. The combined ethyl
acetate extracts are analysed by gas chromatography, mass
spectrometry and NMR and it is found that the major
products were (2R,4R,5R)-2,4-dimethyl-3-keto-5-hydroxy-n-
hexanoic acid δ_lactone and (2R,4R,5R)-2,4-dimethyl-3-
keto-5-hydroxy-n-heptanoic acid δ lactone in total yields
of 20 mg/L for each lactone.

Example 44

For the construction of an S. erythraea strain that
produces ketolides, the construction of plasmid pKETO
required the construction of the following intermediate
plasmids (Fig 23).

Construction of p1-0

The 1.9 kbp segment of pUC1-0 from nucleotide 8715 to
10645 was amplified by PCR using as primers the synthetic
oligonucleotides:
5'-CCCCTGCAGCCGGACCGCACCACCCCTCGTGACGA-3' and
5'-CTTCTAGACTATGAATTCCCTCCGCCCAGC and the DNA of pUC1-0
as template. The PCR product was end repaired and
ligated with plasmid pUC18, which had been linearised by
digestion with SmaI and treated with alkaline
phosphatase. The ligation mixture was used to transform
E. coli DH10B and individual colonies were checked for
their plasmid content. The desired plasmid designated

p1-0 was identified by restriction analysis and DNA
sequencing.

Construction of pX3

The 60bp segment of eryAIII from nucleotide 7006 to 7066
was amplified by PCR using as primers the synthetic
oligonucleotides:

5'-GGCGGAACGTCTTCCCGGCGGCACCT-3' and
5'-CCCCTGCAGCCAGTACCGCTGGGGCTCGAA-3' and pEXDB3 (Roberts,
G. A., et al. (1993) Eur. J. Biochem. 214:305-311) as
template. The PCR product was end-repaired and ligated
with plasmid pUC18, which had been linearised by
digestion with SmaI and then treated with alkaline
phosphatase. The ligation mixture was used to transform
E. coli DH10B and individual colonies were checked for
their plasmid content. The desired plasmid designated
pD3P was identified by restriction analysis and DNA
sequencing.

Construction of pT3

The 0.1 kbp EcoRI and PstI restriction fragment from pX3
was ligated with EcoRI and PstI digested pT7-18. The
ligation mixture was used to transform E. coli DH10B and
individual colonies were checked for their plasmid
content. The desired plasmid designated pT3 was
identified by restricition analysis.

Construction of pT31-0

The 1.9 kbp PstI and fragment from p1-0 was ligated to
PstI digested pT3. The ligation mixture was used to
transform E.coli DH10B and individual colonies were
checked for their plasmid content. The desired plasmid
designated pT31-0 was identified by restriction analysis.

87

Construction of pD31-0

The 3.3 kbp XmnI restriction fragment from pEXDB3
(Roberts, G. A., et al. (1993) Eur. J. Biochem. 214:305-
311) was substituted for the 2.7 kbp XmnI restriction
fragment of pT31-0. The desired plasmid pD31-0 was
identified by restriction analysis.

Construction of pKETO

Plasmid pD31-0 was digested with BglII and the 11.3 kbp
fragment was ligated to pIJ702 which had been linearised
by digestion with BglII. The ligation mixture was used
to transform E. coli DH10B and individual colonies were
check for plasmid content. The desired plasmid
designated pKETO was identified by restriction analysis.

Example 45

Construction of S. erythraea NRRL2338/pKETO

Approximately 5 μg of plasmid pKETO isolated from E. coli
DH10B was used to transform protoplasts of S. erythraea .
NRRL2338 and stable thiostrepton resistant colonies were
isolated. From several such colonies, total DNA was
obtained and analysed by Southern hybridisation, to
confirm that the plasmid had integrated specifically into
the eryAIII gene by homologous recombination. One such
clone was selected and designated S. erythraea
NRRL2338/pKETO.

(ii) Production of ketolides using S. erythraea
NRRL2338/pKETO

S. erythraea NRRL2338/pKETO was inoculated into sucrose-
succinate medium containing 10 μg/ml thiostrepton and
allowed to grow for four days at 30°C. After this time

88

the broth is filtered to remove mycelia, the supernatant adjusted to pH 9.5 and then extracted twice with equal volumes of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness, the residue taken up in methanol (5 mL) and then analysed by HPLC and electrospray MS. It is found that the major product is the expected 3-ketolide in an approximate yield of 10 mg/L. Analysis of the electrospray mass spectrum shows that the proton adduct for this compound displays a MH^+ mass of 558.4, which was confirmed by accurate mass analysis; MH^+ requires 558.36418 $C_{29}H_{52}O_9N$, observed 558.36427.

89

Synthesis of Ketolides by ΔKR6
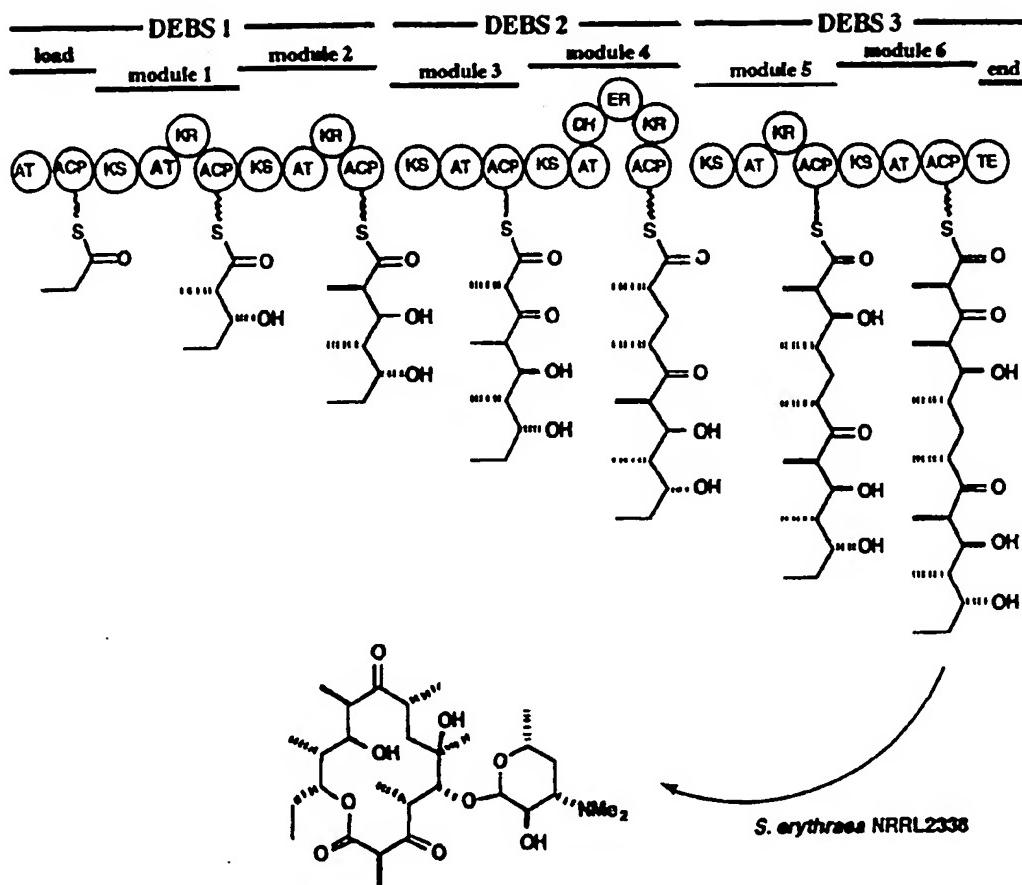
ESMS : MH⁺ = 558.4

C₂₈H₅₂O₉N requires 558.36418; found 558.36427

90

Example 47
Construction of plasmid pMO7

Plasmid pMO7 (like plasmid pMO107 also herein described)
is an SCP2*-based plasmid containing a PKS gene
comprising the ery loading module, the first and second
extension modules of the ery PKS and the ery chain-
terminating thioesterase, except that the DNA segment
encoding the methylmalonyl-CoA:ACP acyltransferase within
the first ery extension module has been specifically
substituted by the DNA encoding the malonyl-CoA:ACP
acyltransferase of module 13 of the rap PKS. It was
constructed via several intermediate plasmids as follows
(Figure 24).

Construction of plasmid pMO1

The approximately 1.3 kbp DNA segment of the eryAI gene
of S. erythraea extending from nucleotide 1948 to
nucleotide 3273 of eryAI (Donadio, S. et al. Science
(1991) 252:675-679) was amplified by PCR employing as
primers the synthetic oligonucleotides:
5'-CATGCTCGAGCTCTCCTGGGAAGT-3' and
5'-CAACCCTGGCCAGGGAAGACGAAGACGG-3', and plasmid pNTEP2
(Example 5) as template. The PCR product was end-
repaired and ligated with plasmid pUC18, which had been
linearised by digestion with SmaI and then treated with
alkaline phosphatase. The ligation mixture was used to
transform E. coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid
pMO1 (3.9 kbp), in which the StuI site bordering the
insert is adjacent to the HindIII site in the polylinker,
was identified by its restriction pattern.

Construction of plasmid pMO2

91

The approximately 0.85 kbp DNA segment of the rapA gene
of S. hygroscopicus, extending from nucleotide 1643 to
nucleotide 2486 of rapA, was amplified by PCR employing
as primers the following oligonucleotides:

5'-TTCCCTGGCCAGGGGTCGCAGCGTG-3' and
5'-CACCTAGGACCGCGGACCACTCGAC-3', and the DNA from the
recombinant bacteriophage _-1E (Schwecke, T. et al. Proc.
Natl. Acad. Sci. USA (1995) 92:7839-7843) as the
template. The PCR product was end-repaired and ligated
with plasmid pUC18, which had been linearised by
digestion with SmaI and then treated with alkaline
phosphatase. The ligation mixture was used to transform
E. coli TG1 recO and individual colonies were checked for
their plasmid content. The desired plasmid pMO2 (3.5
kbp) was identified by its restriction pattern.

Construction of plasmid pMO3

The approximately 1.7 kbp DNA segment of the eryAI gene
of S. erythraea extending from nucleotide 4128 to
nucleotide 5928 of eryAI, was amplified by PCR employing
as primers the synthetic oligonucleotides:
5'-TGGCCAGGGAGTCGGTGCACCTAGGCA-3' and
5'-GCCGACAGCGAGTCGACGCCGAGTT-3' and plasmid pNTEP2 as
template. The PCR product was end-repaired and ligated
with plasmid pUC18, which had been linearised by
digestion with SmaI and then treated with alkaline
phosphatase. The ligation mixture was used to transform
E. coli TG1 recO and individual colonies were checked for
their plasmid content. The desired plasmid pMO3 (4.4
kbp), in which the BalI and AvrII sites are adjacent to
the HindIII site of the polylinker, was identified by its
restriction pattern.

Construction of plasmid pMO4

Plasmid pMO1 was digested with HindIII and BalI and the
1.3 kbp insert was ligated with plasmid pMO3 which had
been digested with HindIII and BalI. The ligation
mixture was used to transform E. coli TG1 recO and
individual colonies were checked for their plasmid
content. The desired plasmid pMO4 (5.6 kbp) was
identified by its restriction pattern.

Construction of plasmid pMO5

Plasmid pMO4 was digested with StuI and the 3.0 kbp
insert was ligated with plasmid pNTEP2 which had been
digested with StuI and the vector purified by gel
electrophoresis to remove the 3.8 kbp insert. The
ligation mixture was transformed into E. coli TG1 recO
and individual colonies were checked for their plasmid
content. The desired plasmid pMO5 (12.8 kbp) was
identified by its restriction pattern.

Construction of plasmid pMO6

Plasmid pMO2 was digested with BalI and AvrII and the
insert was ligated with plasmid pMO5 which had been
digested with BalI and AvrII. The ligation mixture was
used to transform E. coli TG1 recO and individual
colonies were checked for their plasmid content. The
desired plasmid pMO6 (13.5 kbp) was identified by its
restriction pattern.

Construction of plasmid pMO7

Plasmid pMO6 was digested with NdeI and XbaI and the
insert was ligated with plasmid pRM52 (Example 4) which
had been digested with NdeI and XbaI and purified by gel
electrophoresis. The ligation mixture was transformed
into E. coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid

pMO7 (also designated pRMAT2) was identified by its
restriction pattern.

Example 48
Construction of S. coelicolor CH999/pMO7 and production
of TKL derivatives

(i) Construction
Plasmid pMO7 which had been isolated from E. coli ET12567
(MacNeil, D. J. et al. Gene (1992) 111:61-68) was
transformed into protoplasts of S. coelicolor CH999 and
stable thiostrepton resistant colonies were isolated.
Individual colonies were checked for their plasmid
content and the presence of plasmid pMO7 was confirmed by
its restriction pattern.

(ii) Production and isolation of 4-nor-TKL and (Ac)4-nor-
TKL using S. coelicolor CH999/pMO7

S. coelicolor CH999/pMO7 was inoculated into YEME medium
containing 50 _g/ml thiostrepton and allowed to grow for
five days at 28-30%C. After this time the broth was
filtered to remove mycelia and the pH adjusted to pH 3.
The broth was extracted twice with two volumes of ethyl
acetate and the combined ethyl acetate extracts were
washed with an equal volume of saturated sodium chloride,
dried over anhydrous sodium sulphate, and the ethyl
acetate was removed under reduced pressure, to give about
200 mg crude product. This was digested with 2 ml of
methanol, and mixed with 0.5 g of dry silica gel, and
then subjected to flash chromatography on a column of the
same material (1 cm x 15 cm) The column was eluted with
diethyl ether, and fractions of 10 ml each were
collected. Fractions 4-8 were pooled, and the diethyl
ether was evaporated to leave about 10 mg of oily residue
containing the compounds of interest. These were
purified further by hplc on an octadecylsilica reverse

phase column (10 mm x 25 cm) eluted at a flow rate of 2
ml/minute first with an isocratic mixture of
water/methanol 75:25 (vol/vol) for five minutes, then
with a linear gradient of increasing methanol, reaching
water/methanol 55/45 (vol/vol) after 30 minutes. After
about 11 minutes, fractions were collected containing, as
the minor component, (Ac)4-nor-TKL (R_1=Me, R_2=H, R_3=Me)
and after about 18 minutes fractions were collected
containing, as the major component, 4-nor-TKL (R_1=Me,
R_2=H, R_3=Et).

The ^{1}H spectrum of 4-nor-TKL was determined using a Bruker
AM-400 NMR spectrometer. Found: H (400 MHz, $CDCl_3$) 4.18
(1H, dtd, 11.8, 6.1, 2.9 Hz, H-5), 3.75 (1H, ddd, 11.0,
10.0, 4.0 Hz, H-3), 2.35 (1H, dq, 10.0, 7.0 Hz, H-2),
2.20 (1H, ddd, 13.3, 4.0, 2.9 Hz, H-4eq), 1.6 - 1.88 (3H,
m, 2xH-6, H-4ax), 1.41 (1H, d, 7.0 Hz, CH3-3'), 1.01 (1H,
t, 7.5 Hz, CH3-7) ppm.

The ^{13}C NMR spectrum of 4-nor-TKL was also determined (100
MHz, $CDCl_3$): 173.3 (C-1), 77.7 (C-5), 70.4 (C-3), 45.1 (C-
2), 37.7 (C-4), 28.8 (C-6), 13.5 (C-3'), 9.1 (C-7).

Example 49

Construction of plasmid pMO107 and production of TKL
derivatives

(i) Construction

95

Plasmid pMO6 was digested with NdeI and XbaI and the
insert was ligated with plasmid pCJR101 (Example 2) which
had been digested with NdeI and XbaI and purified by gel
electrophoresis. The ligation mixture was transformed
into E. coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid
pMO107 was identified by its restriction pattern.

(ii) Production and isolation of 4-nor-TKL and (Ac)4-nor-
TKL using S. erythraea JC2/pMO107

S. erythraea JC2/pMO107 was prepared by standard
techniques (c.f. Example 26(i)) and inoculated into
sucrose-succinate medium containing 50 _g/ml thiostrepton
and allowed to grow for three-five days at 28-30%C.
After this time the broth was filtered to remove mycelia
and the pH adjusted to pH 3. The broth was extracted
three times with quarter volumes of ethyl acetate and the
combined ethyl acetate extracts were dried over anhydrous
sodium sulphate, and the ethyl acetate was removed under
reduced pressure, to give about 10 mg/L crude product.
This was digested with 2 ml of methanol, and mixed with
0.5 g of dry silica gel, and then subjected to flash
chromatography on a column of the same material (1 cm x
15 cm) The column was eluted with diethyl ether, and
fractions of 10 ml each were collected. Fractions 4-8
were pooled, and the diethyl ether was evaporated to
leave about 15 mg of oily residue containing the
compounds of interest. These were purified further by
hplc on an octadecylsilica reverse phase column (10 mm x
25 cm) eluted at a flow rate of 2 ml/minute first with an
isocratic mixture of water/methanol 75:25 (vol/vol) for
five minutes, then with a linear gradient of increasing
methanol, reaching water/methanol 55/45 (vol/vol) after
30 minutes. After about 11 minutes, fractions were
collected containing, as the minor component, (Ac)4-nor-
TKL and after about 18 minutes fractions were collected

96

containing, as the major component, 4-nor-TKL.

The ¹H and ¹³C spectra of the purified 4-nor-TKL and
(Ac)4-nor-TKL were identical with the spectra obtained
for authentic material.

Example 50

Construction of plasmid pCJR26

Plasmid pCJR26 is an SCP2* based plasmid containing
a PKS gene comprising the ery loading module, the
first and second extension modules of the ery PKS
and the ery chain-terminating thioesterase, except
that the DNA segment encoding the methylmalonyl-
CoA:ACP acyltransferase within the first extension
module has been specifically substituted by the DNA
encoding the malonyl-CoA:ACP acyltransferase of
module 2 of the rap PKS. It was constructed as
follows (Fig 25):
Plasmid pMO6 was digested with NdeI and XbaI and the
insert was ligated with plasmid pCJR24, which had
been digested with NdeI and XbaI and purified by gel
electrophoresis. The ligation mixture was
transformnd into E.coli TG1 rec0 and individual
colonies were checked for their plasmid content. The
desired plasmid pCJR26 was identified by its
restriction pattern.

Example 51

Construction of S. erythraea JC2/pCJR26 and
production of TKL derivatives.

Plasmid pCJR26 was used to transform S.erythraea JC2
protoplasts. Thiostrepton resistant colonies were
selected on R2T20 medium containing 10 µg/ml of

thiostrepton. Several clones were tested for the
presence of pCJR26 integrated into the chromosome,
by Southern blot hybridisation of their genomic DNA
with DIG-labelled DEBS1-TE gene.

A clone with an integrated copy of pCJR26 was grown
in SSM medium, containing 5 μg/ml of thiostrepton
and allowed to grow for seven days at 28-30'C. After
thIs time the broth was filtered to remove mycelia
and the pH was adjusted to pH=3. The broth was
extracted twice with two volumes of ethyl acetate
and the combined ethyl acetate extracts were washed
with an equal volume of saturated sodium chloride,
dried over anhydrous sodium sulphate, and the ethyl
acetate was removed under reduced pressure, to give
about 500 mg of crude product. The products were
shown to be (Ac)4-nor-TKL and 4-nor-TKL:
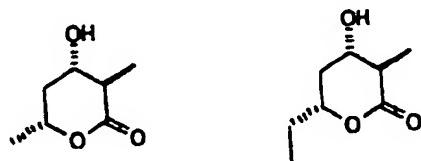
Example 52

Construction of S. erythraea NRRL 2338/pCJR26 and
its use in production of 14-membered macrolides

Approximately 5μg pCJR49 DNA was used to transform
S. erythraea NRRL2338 protoplasts to give a strain
in which the plasmid is integrated into the
chromosome. From several colonies, total DNA was
obtained and analysed by Southern hybridisation to

98

confirm that the plasmid has integrated in module 2
of EryAI to give a novel macrolide biosynthetic
pathway. Further integrations had occurred to give
repeated plasmid sequences.   S. erythraea NRRL
2338 /pCJR49 was inoculated into tryptic soy broth
containing 5µg/ml thiostrepton and incubated at
30°C for three days. 100 ml of this seed culture
was used to inoculate 2 litres of sucrose succinate
defined medium containing 5µg/ ml thiostrepton in 5x
2 litre flasks each containing 500ml medium with 2
springs to aid dispersion and shaken at 300 rpm.
After a further 5 days of growth the cultures were
centrifuged and the pH of the supernatant adjusted
to pH 9. The supernatant was then extracted three
times with an equal volume of ethyl acetate and the
solvent removed by evaporation. Products were
analysed by HPLC/MS and two macrolides were
identified as the erythromycin analogues:

Example 53

99
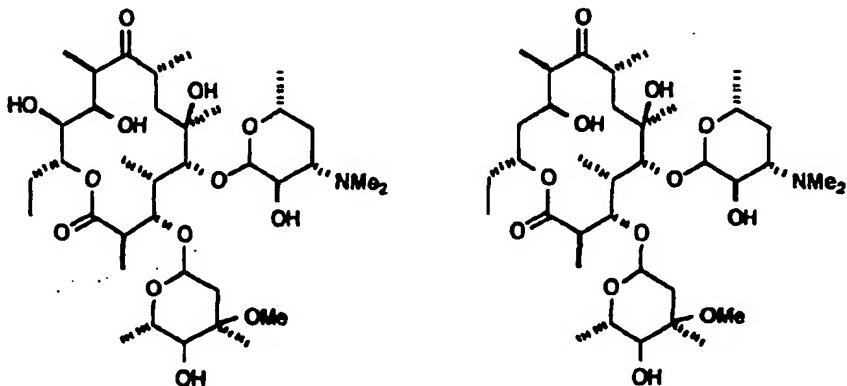
Construction of plasmid pC-ATX

Plasmid pC-ATX is an SCP2* based plasmid containing
a PKS gene comprising the ery loading module, the
first and second extension modules of the ery PKS
and the ery chain-terminating thioesterase, except
that the DNA segment encoding the methylmalonyl-
CoA:ACP acyltransferase within the first extension
module has been specifically substituted by the DNA
encoding the malonyl-CoA:ACP acyltransferase from a
putative type I PKS gene cluster cloned from
Streptomyces cinnamonensis ATCC 14513 (producer of
the polyether polyketide monensin). It was
constructed via several intermediate plasmids as
follows (Figure 26).

Isolation of cosmid pSCIN02.
Genomic library of Streptomyces cinnamonensis ATCC
14513 (the monensin producer) was constructed from
size fractioned 35 - 45 kbp Sau3A fragments of
chromosomal DNA ligated into BamHI-linearised and
alkaline phosphatase- treated cosmid vector pWE15.
The ligation mixture was packaged into λ-particles
using Gigapack packaging extracts, and transfected
into E.coli NM1blue. Approximately 600 colonies of
the library were grown on the surface of a nylon
membrane, lysed, and their DNA was crosslinked to
the membrane by UV irradiation. The membrane was
subsequently used for the screening procedure. The
insert of pMO8 comprising the ketosynthase domain
from module 2 of DEBS was labelled by random
priming in the presence of ^{33}PαATP and used as a
probe for DNA hybridisation. The probe was
hybridised for 16h at 68°C in 4.0xSSC buffer and
subsequently washed off for 1h at 68°C in 0.8xSSC
buffer. Three positive clones were isolated. DNA of
the inserts of all three clones was end sequenced

from T3 and T7 priming sites present in the vector
pWE15. A region homologous to type I ketosynthase
and malonyl-CoA:ACP acyltransferase domains was
discovered in the DNA sequence from the T7 priming
site using clone 2 (named pSCIN02) as a template.
Partial DNA sequencing of the malonyl-CoA:ACP
acyltransferase domain (named ATX) revealed an
unusual sequence motif in the putative substrate
recognition part of the domain wich was
substantially different from previously described
malonate- or methylmalonate-specific CoA:ACP
acyltransferases (Haydock, S.F. et al., FEBS (1995)
374:246-248)

Construction of plasmid pMO38
The approximately 0.9 kbp DNA segment of the ATX
domain was amplified by PCR employing as primers the
following oligonucleotides:
5' CTGGCCAGGGCGCGCAATGGCCGAGCAT 3' and
5' CCCTAGGAGTCGCCGGCAGTCCAGCGCGGCGCCC 3' using the
DNA from the cosmid pSCIN02 as the template. The PCR
product was end-repaired and ligated with plasmid
pUC18, which had been linearised by digestion with
SmaI and then treated with alkaline phosphatase. The
ligation mixture was used to transform E. coli TG1
recO and individual colonies were checked for their
plasmid content. The desired plasmid pMO33 (3.5 kbp)
was identified by its restriction pattern.

Construction of plasmid pMO34
Plasmid pMO34 is a derivative of pMO6 with a
polycloning site inserted after the stop codon of
the inserted D1-AT2 gene. Plasmid pMO6 was digested
wih EcoRI and HindIII and annealed with two
oligonucleotides forming the double-stranded region
of the polycloning site:

           5' AATTCATAACTAGTAGGAGGTCTGGCCATCTAGA 3'

and 5' TCGAAGATCTACCGGTCTGGAGGATGATCAATAC 3'.
The mixture was ligated and transformed into E. coli
TG1 recO. Individual colonies were checked for their
plasmid content. The desired plasmid pMO34 (13.5
kbp) was identified by its restriction pattern.

Construction of plasmid pMO35
Plasmid pMO35 is a derivative of pMO34 containing
TKLS-AT2 gene and a translationally coupled
crotonyl-CoA-reductase gene from Streptomyces
collinus (Wallace et al., E. J. Biochem. (1995) 233:
954-962). The crotonyl-CoA-reductase gene was
excised from the plasmid pZYB3 (the gift of Prof. K.
Reynolds) as an NdeI - BamHI fragment, which was
treated with mung bean nuclease to produce blunt
ends and ligated into pMO34 previously cut with SpeI
and likewise blunt-ended using mung bean nuclease.
The ligation mixture was used to transform E. coli
TG1 recO and individual colonies were checked for
their plasmid content. The desired plasmid pMO35
(14.2 kbp), with the correct orientation of the
crotonyl-CoA-ketoreductase gene, was identified by
its restriction pattern.

Construction of plasmid pMO36
Plasmid pMO33 was digested with BalI and AvrII and the
insert was ligated with plasmid pMO35 which had been
digested with BalI and AvrII. The ligation mixture was
used to transform E.coli TG1 recO and individual colonies
were checked for their plasmid content. The desired
plasmid pMO36 (13.5 kbp) was identified by its
restriction pattern.

Example 54

Construction of plasmid pC-ATX

102

Plasmid pMO36 was digested with NdeI and XbaI and
the insert was ligated with plasmid pCJR29, which
had been digested with NdeI and XbaI and purified by
gel electrophoresis. The ligation mixture was
transformed into E.coli TG1 recO and individual
colonies were checked fon their plasmid content. The
desired plasmid pC-ATX was identified by its
restriction pattern.

Example 55
Construction of S. erythraea JC2/pC-ATX and
production of TKL derivatives.

Plasmid pC-ATX was used to transform S.erythraea JC2
protoplasts. Thiostrepton resistant colonies were
selected on R2T20 medium containing 10 µg/ml of
thiostrepton. Several clones were tested for
presence of pC-ATX integrated into the chromosome,
by Southern blot hybridisation of their genomic DNA
with DIG-labelled DNA encoding the DEBS1-TE gene.
A clone with an integrated copy of pC-ATX was grown
in SSM medium, containing 5 µg/ml of thiostrepton,
and allowed to grow for seven days at 28-30'C. After
thIs time the broth was filtered to remove mycelia
and the pH adjusted to pH=3. The broth was extracted
twice with two volumes of ethyl acetate and the
combined ethyl acetate extracts were washed with an
equal volume of saturated sodium chloride, dried
over anhydrous sodium sulphate, and the ethyl
acetate was removed under reduced pressure, to give
about 500 mg of crude product. The products were
characterised by gas chromatography, mass
spectrometry and NMR, and were shown to be (2S, 3R,
4S, 5R)-2-methyl-4-ethyl-3,5-dihydroxy-n-hexanoic
acid δ-lactone and (2S, 3R, 4S, 5R)-2-methyl-4-
ethyl-3,5-dihydroxy-n-heptanoic acid δ-lactone:
*

103

Example 56
Construction of S. erythraea NRRL 2338/pC-ATX and its
use in production of 14-membered macrolides

Approximately 5μg pC-ATX DNA was used to transform
S. erythraea NRRL2338 protoplasts to give a strain
in which the plasmid is integrated into the
chromosome. From several colonies, total DNA was
obtained and analysed by Southern hybridisation to
confirm that the plasmid has integrated in module 2
of EryAI to give a novel macrolide biosynthetic
pathway. Further integrations had occurred to give
repeated plasmid sequences.   S. erythraea
NRRL2338 /pC-ATX was inoculated into tryptic soy
broth containing 5μg/ml thiostrepton and incubated
at 30°C for three days. 100 ml of this seed
culture was used to inoculate 2 litres of sucrose
succinate defined medium containing 5μg/ ml
thiostrepton in 5x 2 litre flasks each containing
500ml medium with 2 springs to aid dispersion and
shaken at 300 rpm. After a further 5 days of growth
the cultures were centrifuged and the pH of the
supernatant adjusted to pH 9. The supernatant was
then extracted three times with an equal volume of
ethyl acetate and the solvent removed by
evaporation. Products were analysed by HPLC/MS and
two macrolide products were identified:
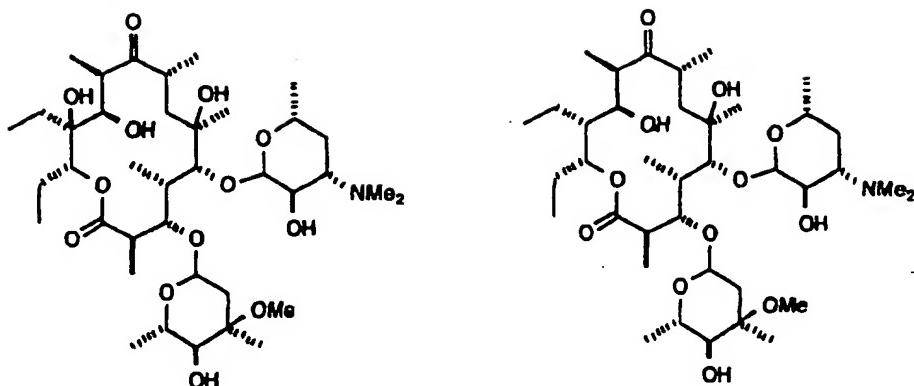
Example 57a

Construction of plasmid pC-AT12

Plasmid pC-AT12 is an SCP2* based plasmid containing
a PKS gene comprising the ery loading module, the
first and second extension modules of the ery PKS
and the ery chain-terminating thioesterase, except
that the DNA segment encoding the methylmalonyl-
CoA:ACP acyltransferase within the second extension
module has been specifically substituted by the DNA
encoding the malonyl-CoA:ACP acyltransferase of
module 2 of the rap PKS. It was constructed via
several intermediate plasmids as follows (Figure
27).

Construction of plasmid pMO25.

The approximately 1.0 kbp DNA segment of the eryAI
gene of S.erythraea extending from nucleotide 6696
to nucleotide 7707 of eryAI (Donadio. S. et al.,
Science (1991) 252, 675-679) was amplified by PCR
employing as primers synthetic oligonucleotides:
5' GGCGGGTCCGGAGGTGTTCACCGAGTT 3'
and 5' ACCTTGGCCAGGGAAGACGAACACTGA 3', and plasmid

pNTEp2 as a template. The PCR product was end-
repaired and ligated with plasmid pUC18, which had
been linearised by digestion with SmaI and then
treated with alkaline phosphatase. The ligation
mixture was used to transform E.coli TG1 recO and
individual colonies were checked for their plasmid
content. The desired plasmid pMO25 (3.6 kbp), in
which the StuI site bordering the insert is adjacent
to the HindIII site in the polylinker, was
identified by its restriction pattern.

Construction of plasmid pMO26
The approximately 0.6 kbp DNA segment of the eryAI
gene of S. erythraea extending from nucleotide 8660
to nucleotide 9258 of eryAI, was amplified by PCR
employing as primers the synthetic oligonucleotides:
5' TCCTAGGCCGGGCCGGACTGGTCGACCTGCCGGGTT 3'
and 5' AAACACCGCGACCTGGTCCTCCGAGC 3', and plasmid
pNTEP2 as template. The PCR product was end-repaired
and ligated with plasmid pUC18, which had been
linearised by digestion with SmaI and then treated
with alkaline phosphatase. The ligation mixture was
used to transform E. coli TG1 recO and individual
colonies were checked for their plasmid content. The
desired plasmid pMO26 (3.2 kbp), in which the AvrII
site is adjacent to the HindIII site of the
polylinker, was identified by its restriction
pattern.

Construction of plasmid pMO27.
Plasmid pMO25 was digested with EcoRI and BalI and
the 1.0 kbp insert was ligated with plasmid pMO2
which had been digested with EcoRI and BalI. The
ligation mixture was used to transform E. coli TG1
recO and individual colonies were checked for their
plasmid content. The desired plasmid pMO27 (4.4 kbp)
was identified by its restriction pattern.

106

Construction of plasmid pMO32.

Plasmid pMO26 was digested with AvrII and HindIII and the 0.6 kbp insert was ligated with plasmid pMO27 which had been digested with AvrII and HindIII. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pMO32 (5.1 kbp) was identified by its restriction pattern.

Constrnction of plasmId pMO33.

Plasmid pMO32 was digested with BspEI and SexAI and the 2.7 kbp insert was ligated with plasmid pNTEP2 which had been digested with the same two enzymes and purified by gel electrophoresis to remove the 2.8 kbp insert. The ligation mixture was transformed into E.coli TG1 recO and individual colonies were checked for their plasmid content. The plasmid pMO33 (12.8 kbp) was identified by its restriction pattern.

Example 57b
Construction of plasmid pC-AT12.

Plasmid pMO33 was digested with NdeI and XbaI and the insert was ligated with plasmid pCJR29, which had been digested with NdeI and XbaI and purified by gel electrophoresis. The ligation mixture was transformnd into E.coli TG1 rec0 and individual colonies were checked for their plasmid content. The desired plasmid pC-AT12 was identified by its restriction pattern.

Example 58a
Construction of S.erythraea JC2/pC-AT12 and production of TKL derivatives.

Plasmid pC-AT12 was used to transform S.erythraea

JC2 protoplasts. Thiostrepton resistant colonies were selected on R2T20 medium containing 10 μg/ml of thiostrepton. Several clones were tested for the presence of pC-AT12 integrated into the chromosome, by Southern blot hybridisation of their genomic DNA with DIG-labelled DNA encoding the DEBS1-TE gene. A clone with an integrated copy of pC-AT12 was grown in SSM medium, containing 5 μg/ml of thiostrepton and allowed to grow for seven days at 28-30'C. After thIs time the broth was filtered to remove mycelia and the pH adjusted to pH=3. The broth was extracted twice with two volumes of ethyl acetate and the combined ethyl acetate extracts were washed with an equal volume of saturated sodium chloride, dried over anhydrous sodium sulphate, and the ethyl acetate wass removed under reduced pressure, to give about 500 mg of crude product. The products were shown to be (3R, 4S, 5R)-4-methyl-3,5-dihydroxy-n-hexanoic acid δ-lactone and (3R, 4S, 5R)-4-methyl-3,5-dihydroxy-n-heptanoic acid δ-lactone:

Example 58b
Construction of S. erythraea NRRL 2338/pC-AT12 and its use in production of 14-membered macrolides

Approximately 5μg pC-AT12 DNA was used to transform S. erythraea NRRL2338 protoplasts to give a strain in which the plasmid is integrated into the chromosome. From several colonies, total DNA was obtained and analysed by Southern hybridisation to

confirm that the plasmid has integrated 3' of module
2 of EryAI to give a novel macrolide biosynthetic
pathway. Further integrations had occurred to give
repeated plasmid sequences. S. erythraea
NRRL2338 /pC-AT12 was inoculated into tryptic soy
broth containing 5µg/ml thiostrepton and incubated
at 30°C for three days. 100 ml of this seed
culture was used to inoculate 2 litres of sucrose
succinate defined medium containing 5µg/ ml
thiostrepton in 5x 2 litre flasks each containing
500ml medium with 2 springs to aid dispersion and
shaken at 300 rpm. After a further 5 days of growth
the cultures were centrifuged and the pH of the
supernatant adjusted to pH 9. The supernatant was
then extracted three times with an equal volume of
ethyl acetate and the solvent removed by
evaporation. Products were analysed by HPLC/MS and
two macrolide products were identified:
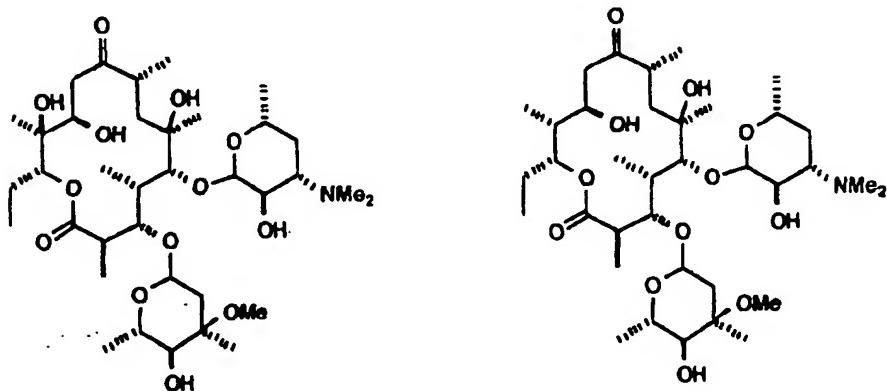
Example 59
Construction of plasmid pCJR49

pCJR49 is a pCJR24-based plasmid containing a mutant
DEBS1-TE gene which has no ketoreductase in module 2,
and the AT domain in module 2 has been replaced by RAPS
AT2 in order to incorporate a malonyl extender instead
of a methylmalonyl extender in the second module
(Figure 28).

pMO32 was digested with BspE I and SexA I and the
fragment containing the AT from RAP module 2 was cloned
into pUC1-0 which had been previously digested with
BspE I and SexA I, to yield the plasmid pCJR43.

pCJR43 was digested with Nde I and Xba I and the
fragment containing the mutant DEBS1-TE gene was cloned
into pCJR24 which had previously been digested with Nde
I and Xba I, to yield plasmid pCJR49. pCJR49 was
confirmed by restriction enzyme mapping.

Example 60
Construction of S. erythraea JC2/pCJR49 and production
of TKL derivatives

i)   Approximately 5μg pCJR49 DNA was used to transform
S. erythraea JC2 protoplasts to give a strain in which
the plasmid is integrated into the chromosome. From
several colonies total DNA is obtained and analysed by
Southern hybridisation to confirm that the plasmid has
integrated into the eryTE. S. erythraea JC2/pCJR49 is
inoculated into tryptic soy broth containing 5μg/ ml
thiostrepton and incubated at 30°C for three days.
100ml of this seed culture was used to inoculate 2
litres of sucrose succinate defined medium containing
5μg/ ml thiostrepton in 5x 2 litre flasks each
containing 500 ml medium with 2 springs to aid
dispersion and shaken at 300 rpm. After a further 5
days of growth the cultures were centrifuged and the pH
of the supernatant was adjusted to pH 3. The
supernatant was then extracted three times with an
equal volume of ethyl acetate and the solvent removed

110

by evaporation. Products were dissolved in methanol
and analysed by GCMS on a Finnegan-MAT GCQ System.
This analysis indicated that by comparison to synthetic
standards two new lactones were present. These
products were (4S,5R)-4-methyl-3-keto-5-hydroxyhexanoic
acid δ lactone and (4S,5R)-4-methyl-3-keto-5-
hydroxyheptanoic acid δ lactone:

Example 61
Construction of S. erythraea NRRL 2338/pCJR49 and its
use for production of 14-membered macrolides

5μg pCJR49 DNA was used to transform S. erythraea
NRRL2338 protoplasts to give a strain in which the
plasmid is integrated into the chromosome. From
several colonies total DNA is obtained and analysed by
Southern hybridisation to confirm that the plasmid has
integrated in module 2 of EryAI to give a novel
macrolide biosynthetic pathway. Further integrations
had occurred to give repeated plasmid sequences.   S.
erythraea /pCJR49 is inoculated into tryptic soy broth
containing 5μg/ml thiostrepton and incubated at 30°C
for three days. 100ml of this seed culture was used to
inoculate.2 litres of sucrose succinate defined medium
containing 5μg/ml thiostrepton in 5x 2 litre flasks
each containing 500ml medium with 2 springs to aid
dispersion and shaken at 300rpm. After a further 5
days of growth the cultures were centrifuged and the pH

111

of the supernatant adjusted to pH 9. The supernatant
was then extracted three times with and equal volume of
ethyl acetate and the solvent removed by evaporation.
Products were analysed by HPLC/MS and two macrolides
were identified:
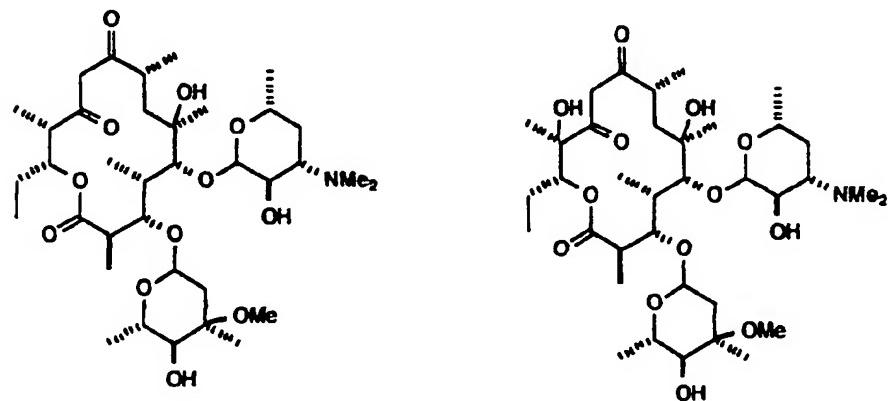
Example 62: Construction of plasmid (Fig. 29A-F)

Plasmid pCART11 is a pRM52-based plasmid containing a PKS
gene comprising the avermectin loading module, modules 5
and 6 of the ery PKS, and the ery chain-terminating
thioesterase. It was constructed via several intermediate
plasmids as follows.

Construction of plasmid pCAR1

Plasmid pARLD was digested with BamHI and BglII and 1.70
kbp insert was ligated with plasmid pEXD3 which had been
digested with BglII. The ligation mixture was used to
transform E.Coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid

pCAR1 was identified by its restriction pattern.

Construction of plasmid pCAR5

The 250 bp DNA segment of the eryAIII gene of S.erythraea
extending from nucleotide 4807 to nucleotide 5052 of
eryAIII, was amplified by PCR employing as primers the
synthetic oligonucleotides:
5' TTTGCTAGCGATCGTCGGCATGGCGTGCCGGTT3'
5'CCCACGAGATCTCCAGCATGATCC3'
The plasmid pEXD3 was used as a template. The PCR product
was end-repaired and ligated with pUC18, which had been
linearised by digestion with SmaI and then treated with
alkaline phosphatase. The ligation mixture was used to
transform E.Coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid
pCAR5 in which the NheI site is adjacent to the EcoRI site
of the polylinker, was identified by its restriction
pattern and sequence analysis.

Construction of plasmid pCAR2

Plasmid pCAR5 was digested with NheI and BglII and 250 bp
insert was ligated with plasmid pCAR1 which had been
digested with NheI and BglII. The ligation mixture was used
to transform E.Coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid
pCAR2 was identified by its restriction pattern.

Construction of plasmid pCAR21

Plasmid pARTr was digested with XbaI and the 1.20 kbp
tetracyclin gene was ligated with plasmid pCAR2 which had
been digested with XbaI. The ligation mixture was used to
transform E.Coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid
pCAR21 was identified by its restriction pattern

113

Construction of plasmid pCART3

Plasmid pCAR21was digested with PacI and PstI and 13.0 kbp insert was ligated with plasmid pRM52 which had been digested with PacI and NsiI. The ligation mixture was used to transform E.Coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pCART3 was identified by its restriction pattern

Construction of plasmid pIGlet

Plasmid pARTr was digested with XbaI and the 1.20 kbp tetracyclin gene was ligated with plasmid pIGI which had been digested with XbaI. The ligation mixture was used to transform E.Coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pIGlet was identified by its restriction pattern.

Construction of plasmid pCART11

Plasmid pCAR21was digested with NheI and 12.0 kbp insert was ligated with plasmid pIGlet which had been digested NheI. The ligation mixture was used to transform E.Coli TG1 recO and individual colonies, resistent to tetracyclin activity were checked for their plasmid content. The desired plasmid pCART11 was identified by its restriction pattern.

Example 63
Construction of S.erythraea NRRL2338/pCART11 and its use for production of triketide lactones

Approximately 5-10 μg of pCART11, isolated from TG1 recO was transformed into S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated.

A 5ml fermentation of S.erythraea NRRL2338/pCART11 was

carried out in TSB medium and after two days at 30°C, the
mycelium was used to inoculate 50 ml of sucrose-succinate
medium containing thiostrepton (50 µg/ml). After growth at
30°C for four days, the whole broth was extracted twice
with an equal volume of ethyl acetate. The solvent was
concentrated and the mixture analysed on the GC-MS. The
following compounds were identified.
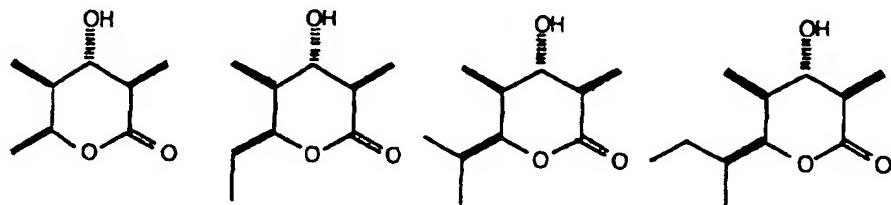
Example 64
Construction of plasmid pARE24

Plasmid pARE24 is a pCJR24-based plasmid containing a PKS
gene comprising the ery loading module, modules 5 and 6 of
the ery PKS, and the ery chain-terminating thioesterase. It
was constructed as follows (Figure 30).

Construction of plasmid pARE24

Plasmid pCAR21 was digested with PacI and XbaI and the 13.0
kbp insert was ligated with plasmid pCJR24 which had been
digested with PacI and XbaI. The ligation mixture was used
to transform E.Coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid
pARE24 was identified by its restriction pattern.

Construction of S.erythraea NRRL2338/pARE24 and its use for
production of triketide lactones

115

Approximately 5-10 μg of pARE24, isolated from TG1 recO was transformed into S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated.

A 5ml fermentation of S.erythraea NRRL2338/pARE24 was carried out in TSB medium and after two days at 30°C, the mycelium was used to inoculate 50 ml of sucrose-succinate medium containing thiostrepton (50 μg/ml). After growth at 30°C for four days, the whole broth was extracted twice with an equal volume of ethyl acetate. The solvent was concentrated and the mixture analysed on the GC-MS. The following compounds were identified.
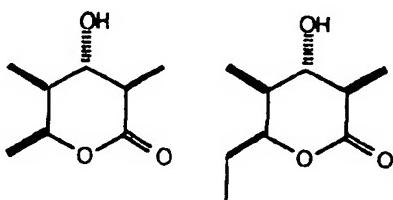
Example 65
Construction of plasmid (Figure 30) pARA24

Plasmid pARA24 is a pCJR24-based plasmid containing a PKS gene comprising the avermectin loading module, modules 5 and 6 of the ery PKS, and the ery chain-terminating thioesterase. It was constructed as follows.

Construction of plasmid pARA24

Plasmid pIG1 was digested with PacI and NheI and 1.70 kbp insert was ligated with plasmid pARE24 which had been digested with PacI and NheI. The ligation mixture was used to transform E.Coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pARA24 was identified by its restriction pattern.

SUBSTITUTE SHEET (RULE 26)

116

Example 66

Construction of S.erythraea NRRL2338/pARA24

Approximately 5-10 μg of pARA24, isolated from TG1 recO was transformed into S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated.

A 5ml fermentation of S.erythraea NRRL2338/pARA24 was carried out in TSB medium and after two days at 30°C, the mycelium was used to inoculate 50 ml of sucrose-succinate medium containing thiostrepton (50 μg/ml). After growth at 30°C for four days, the whole broth was extracted twice with an equal volume of ethyl acetate. The solvent was concentrated and the mixture analysed on the GC-MS. The following compounds were identified.
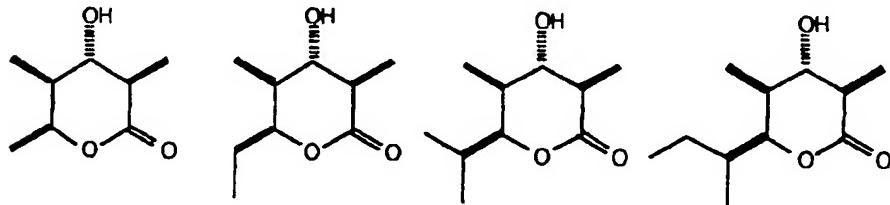
Example 67

Construction of plasmid pARL3

The plasmid pARL3 is a pCJR24-based plasmid containing a PKS gene comprising the ery loading module, modules 5 and 6 of the ery PKS, and the ery thioesterase. The junction between the loading module and the KS5 domain is made at the very N-terminal edge of KS5. It was constructed via several intermediate plasmids as follows (Figure 31):

Construction of plasmid pARL1

117

The 450 bp DNA segment of the eryAI gene of S.erythraea
extending from nucleotide 1 to nucleotide 10631 of eryAI,
was amplified by PCR employing as primers the synthetic
oligonucleotides: (bases   in   bold   letters   denote   the
restriction enzyme sites).

      SphI
5' GGCGGCATGCGGCGGTTCCT3'

     NheI   HpaI
5'AAGCTAGCGGTTCGCCGGGCGCCGCTTCGTTGGTCCGCGCGCGGGTTAAC3'

The plasmid pARE24 was used as a template. The PCR product
was end-repaired and ligated with pUC18, which had been
linearised by digestion with SmaI and then treated with
alkaline phosphatase. The ligation mixture was used to
transform E.coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid
pARL1, in which the NheI site is adjacent to the EcoRI site
of the polylinker, was identified by its restriction
pattern and sequence analysis.

Construction of plasmid pARL2

Plasmid pARL1 was digested with NheI and SphI and the 450
bp insert was ligated with plasmid pARE24 which had been
digested with NheI and SphI. The ligation mixture was used
to transform E.coli TG1 recO and individual colonies were
checked for their plasmid content. The desired plasmid
pARL2 was identified by its restriction pattern.

Construction of plasmid pARL3

The following complementary synthetic oligonucleotides were
synthesised so as when annealed, they would have the
necessary pattern at the 5' and 3' ends that is produced by
the action of HpaI and NheI respectively

SUBSTITUTE SHEET (RULE 26)

5'AACCCGCGCGCGGACCAACGAAGCGGCGCCCGGCGAACCG3'

5'CTAGCGGTTCGCCGGGCGCCGCTTCGTTGGTCCGCGCGCGGGTT3'

The synthetic oligonucleotides were annealed to give double-stranded DNA which was ligated with plasmid pARL2 which had been digested with NheI and HpaI. The ligation mixture was used to transform E. coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pARL3 was identified by its restriction pattern.

Example 68

Construction of S.erythraea JC2-pARL3 and its use for production of triketide lactones

Approximately 5-10 μg of pARL3, isolated from TG1 recO was transformed into S. erythraea JC2 and stable thiostrepton resistant colonies were isolated. A 5 ml fermentation of JC2-pARL3 was carried out in TSB medium and after two days at 30°C, the mycelium was used to inoculate 50 ml of sucrose-succinate medium containing thiostrepton (50 μg/ml). After growth at 30°C for four days, the whole broth was extracted twice with an equal volume of ethyl acetate. The solvent was concentrated and the mixture analysed on the GC-MS. The following compounds were identified:

119
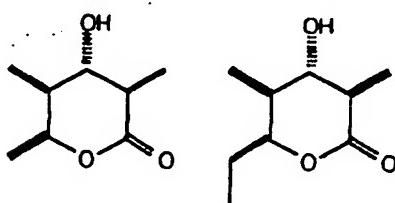
Example 69

Construction of S. erythraea ERMD1, carrying a hybrid PKS gene in which the avr loading didomain is substituted for the ery loading didomain of S. erythraea NRRL 2338

(i) Construction of plasmid pAVLD

Plasmid pCRabc (Example 9) was linearised with BamHI and ligated to pIJ702 previously digested with BglII. The mixture contained the desired plasmid pAVLD (Figure 32). The ligation mixture was transformed into E.coli TG1 recO and individual colonies were checked for their plasmid content. The desired plasmid pAVLD was identified by its restriction pattern (Figure 32).

(ii) Construction of S.erythraea ERM D1

Approximately 5-10 μg of pAVLD, isolated from E. coli TG1recO(pAVLD) was transformed into S. erythraea NRRL2338 and stable thiostrepton resistant colonies were isolated. One of these colonies was selected and total DNA was digested with PstI and analysed by Southern hybridisation employing as a probe the insert from plasmid pCRc which contains the fragment of the ery AI gene encoding the ketosynthase domain KS1. The analysis showed positively-hybridizing PstI fragments of 8.5 kbp, 4.8 kbp and 33 kbp, indicating the presence of two tandemly integrated copies of pAVLD (Figure 33).

Example 70

Isolation of erythromycins altered at C-13

A 50 ml fermentation of S. erythraea ERMD1 was carried out on tap water medium and after 4 days at 30°C the mycelium was harvested and used to inoculate 1.5 litres of sucrose-succinate medium containing thiostrepton (50g/ml).

120

After growth at 30°C for 4 days, the whole broth was extracted twice with an equal volume of ethyl acetate.

The combined extracts were concentrated under reduced pressure and subjected twice to preparative thin layer chromatography on silica plates (20 x 20cm) eluted with chloroform/methanol/.88 ammonia 8:2:0.01 (by vol). The products were separated by hplc on a PhaseSep C18 base-deactivated reverse phase column S5odS (octadecylsilica) 6(4.6mm x 250mm), eluted with methanol/0.5% ammonium acetate (70:30 (vol/vol), at 1 ml.min. Fractions were collected between 7 and 11 minutes from three separate injections, and the pooled fractions were re-injected in ten separate injections. The order of elution from the column was: erythromycin B analogues, followed by erythromycin D analogues and erythromycin A analogues. B and D analogues emerged after 8-10 minutes, erythromycin A analogue 3-4 minutes later. The analogues containing a C-4 (isobutyryl) starter unit are eluted earlier, with the analogues with C-5 (2-methylbutyryl) starter unit emerging several minutes later, although the C-4 late (eryA analogue) and the early C-5 (erythromycins B and D analogue) overlap. High resolution MS gave results for C-4 eryA, eryB and eryD analogues, and for C-5 eryA and eryB analogues, which correspond closely to those calculated:

Analogue	Calc'd Mass	Measured Mass
C5-eryA	762.5004	762.5021
C4-eryA	748.4847	748.4820
C5-eryB	746.4898	748.5077
C4-eryB	732.4898	732.4933

In these experiments natural erythromycins were present only in low or undetectable amounts, and there were no detectable amounts of eryC analogues. The overall concentration ratio of C-4/C-5 compounds in the

SUBSTITUTE SHEET (RULE 26)

121

fermentation broth, as assessed by ESMS of ethyl acetate
extracts of broths, was between 4:1 and 6:1 in favour of
C-4 compounds. The ratio of A:B:D analogues is variable,
about 15:60:25, but with an increasing proportion of A
analogues as the fermentation proceeds. The total yield
of erythromycins is about 400 µg/litre.

Example 71
Construction and use of S. erythraea NRRL2338/pRMTE

(i) Construction

Approximately 5 µg of plasmid pRMTE (Example 6) isolated
from E. coli TGI recO (pRMTE) was transformed into
protoplasts of S. erythraea NRRL2338 and stable
thiostrepton resistant colonies were isolated. One of
these was selected and designated S. erythraea
NRRL2338/pRMTE.

(ii) Enhanced production of erythromycin A and
erythronolides using S. erythraea NRRL2338/pRMTE

S. erythraea NRRL2338/pRMTE was grown in sucrose and
succinate medium containing 50 µg/ml thiostrepton at 28-
30%C. After 3 days the whole broth was extracted twice
with an equal volume of ethyl acetate, the combined ethyl
acetate extracts were washed with saturated sodium
chloride solution, dried over anhydrous sodium sulphate,
and concentrated under reduced pressure.
Examination of the extract by thin layer chromatography
on silica plates eluted with isopropyl
ether:methanol:ammonium hydroxide 75:35:1 (by volume)
showed the presence of several components. Electrospray
mass spectrometry of the extracts revealed the presence
of a mixture of erythromycin A, erythronolide B (EB) and
6-deoxyerythronolide B (6-DEB), together with minor
amounts of (Ac)-6-DEB as its sodium adduct, (Ac)-EB as

122

its Na adduct (411.1), and EB as its Na adduct (424.1), and also TKL (m/e 159.1). The yield of erythromycin A plus erythronolide B was about 500 mg/L of medium, compared to about 50 mg /L produced by S. erythraea NRRL2338 fermented under identical conditions. Cells of S. erythraea NRRL2338/pRMTE harvested from the fermentation broth after 3 days were disrupted and their protein content was examined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. Three high molecular weight bands, corresponding to the erythromycin PKS multienzyme subunits DEBS1, DEBS2 and DEBS3 were observed, approximately ten times more intense than the same protein band seen from cell extracts of S. erythraea NRRL2338 prepared by the same procedure (Caffrey, P. et al. FEBS Letters (1992) 304:225-228).

An identical fermentation of S. erythraea NRRL2338/pRMTE was carried out except that the medium was supplemented with 5 mM potassium propionate. After three days the broth was extracted with ethyl acetate as before, and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate, and concentrated. Preparative TLC using the system isopropyl ether:methanol:ammonium hydroxide 75:35:1 (by volume) separated two major components. Analytical TLC showed that the faster running component (Rf 0.8) has the same mobility as authentic 6-DEB; and the slower migrating material was an approximately equal mixture of a component of Rf 0.63, co-migrating with an authentic sample of TKL; and a component of Rf 0.60, with the same mobility as an authentic sample of EB. Electrospray mass spectrometry (ESMS) on a VG BioQ mass spectrometer operated in positive ion mode showed that the component of Rf 0.75 had m/e 387.4, as required for 6-DEB. ESMS of the mixture of the components with Rf values 0.60 and 0.63 confirmed the presence of TKL and EB.

123

Example 72

Construction and use of S. erythraea TER43/pRMTE

(i) Construction

Approximately 5 μg of plasmid pRMTE was transformed into protoplasts of S. erythraea TER43 (Cortes, J. et al.,Science (1995) 268:1487-1489) and stable thiostrepton resistant colonies were isolated. One of these was selected and designated S. erythraea TER43/pRMTE.

(ii)Enhanced production of TKL using S. erythraea TER43/pRMTE

S. erythraea TER43/pRMTE was inoculated into 1L sucrose-succinate medium and allowed to grow for 3 days at 28-30%C. After 3 days, the broth was extracted twice with an equal volume of ethyl acetate, and the combined ethyl acetate extracts were dried over anhydrous sodium sulphate and concentrated. Analysis of the extract by electrospray mass spectrometry (operated in the positive ion mode) showed the presence of TKL (m/e 173.1) and of (Ac)-TKL (m/e 159.1). The combined yield of triketide lactones was 100 mg/L, compared with 10 mg/L obtained by fermentation of S. erythraea TER43 under identical conditions. Cells of S. erythraea TER43/pRMTE, harvested from the fermentation broth after 3 days, were disrupted and their protein content was examined by sodium dodecyl sulphate/polyacrylamide gel electrophoresis. A high molecular weight band, corresponding to the erythromycin PKS subunit DEBS1 with the attached thioesterase domain (Cortes, J. et al. Science (1995) 268:1487-1489) was observed, approximately ten times more intense than the same protein band seen from cell extracts of S. erythraea prepared by the same procedure.

Example 73

Construction and use of S. erythraea NRRL2338/pCJRTE

(pCJR30)

(i) Construction
Approximately ☐5 g of plasmid pCJRTE (pCJR30) is
transformed into protoplasts of S. erythraea NRRL2338 and
stable thiostrepton resistant colonies are isolated.
From several such colonies, total DNA is obtained and
analysed by Southern hybridisation, to confirm that the
plasmid has integrated specifically into the eryA genes
by homologous recombination, so as to place the resident
eryA genes under the control of the actI promoter derived
from plasmid pCJRTE (pCJR30), while the DEBS1-TE gene
borne by the incoming plasmid is placed by the
integration event under the control of the chromosomal
eryA promoter.

(ii) Enhanced production of erythromycins and their
precursors using S. erythraea NRRL2338/pCJRTE (pCJR30).

S. erythraea NRRL2338/pCJRTE (pCJR30) is inoculated into
sucrose-succinate medium containing 50 μg/ml thiostrepton
and allowed to grow for four days at 30%C. After this
time the broth is filtered to remove mycelia and then
extracted twice with an equal volume of ethyl acetate.
The combined ethyl acetate extracts are analysed by mass
spectrometry and it is found that the mixture contains
erythromycin A, accompanied by 6-DEB, (Ac)-DEB, TKL and
(Ac)-TKL, in total amounts 100 mg/L, or 5 times the total
amount of erythromycins and precursors of erythromycins
that are obtained using S. erythraea NRRL2338 under the
same conditions.

Example 74
Construction and use of S. erythraea JC2/pCJRTE (pCJR30)

(ii) Construction
Approximately 5 _g of plasmid pCJRTE (pCJR30) is

transformed into protoplasts of S. erythraea JC2 and
stable thiostrepton resistant colonies are isolated.
From several such colonies, total DNA is obtained and
analysed by Southern hybridisation, to confirm that the
plasmid has integrated specifically into the portion of
the eryAIII gene that encodes the C-terminal
thioesterase/cyclase, by homologous recombination.

(ii)Enhanced production of triketide lactones using S.
erythraea JC2/pCJRTE (pCJR30)

S. erythraea JC2/pCJRTE (pCJR30) is inoculated into
sucrose-succinate medium containing 50 _g/ml thiostrepton
and allowed to grow for four days at 30%C. After this
time the broth is filtered to remove mycelia and then
extracted twice with an equal volume of ethyl acetate.
The combined ethyl acetate extracts are analysed by mass
spectrometry and NMR and it is found that the major
product is TKL, and the minor product (Ac)TKL, in total
yields (100 mg/L) 10 fold greater than obtained using S.
erythraea TER43.

Example 75
Construction and use of S. erythraea NRRL2338/pIG1

(i) Construction
Approximately 5 _g of plasmid pIG1 is transformed into
protoplasts of S. erythraea NRRL2338 and stable
thiostrepton resistant colonies are isolated. From
several such colonies, total DNA is obtained and analysed
by Southern hybridisation, to confirm that the plasmid
has integrated specifically into the portion of the
eryAIII gene that encodes the C-terminal
thioesterase/cyclase, by homologous recombination.

(ii) Production of 14-membered lactones using S.
erythraea NRRL/pIG1

S. erythraea NRRL/pIG1 is inoculated into tap water
medium containing 50 _g/ml thiostrepton and allowed to
grow for four days at 30%C. After this 20 ml of the
mycelium is used to seed 500 ml of sucrose-succinate
medium containing 50 µg/ml thiostrepton, in a 2L flask
with a single spring to reduce clumping, shaken at 280
rpm. After between 3.5 and 6 days, the broth is filtered
to remove mycelia and then extracted three times with a
quarter volume of ethyl acetate. The combined ethyl
acetate extracts are dried over anhydrous sodium sulphate
and solvent removed by evaporation. Analysis of the
product mixture using GC and electrospray MS revealed
that of a total of 5-6 mg/L of 14-membered macrolide
products, the major component was (s-pent)'-erythromycin D
(about 1.5 mg/L), with other components present being (s-
pent)-erythromycin B and (s-pent)-erythromycin A; (i-
but)-erythromycins A, B and D; and small amounts of
natural erythromycins A, B and D. The extracts also
contained significant amounts (11 mg/l) of TKL's: (s-
pent)-TKL (5mg/l), (i-but)-TKL and TKL. (NB s-pent and
i-but indicate 1-methylpropyl and isopropyl side-chains,
respectively, corresponding to the use of s-2-methylbutyl
and i-butanoyl starter substrates.)

Example 76
Determination of antibiotic activity of novel
erythromycin A analogues

A 3 ml overnight culture of Bacillus subtilis ATCC 6633
was grown at 30%C in nutrient broth (Difco). 200ml of
nutrient 1.5% agar (difco) at 46%C was seeded with 1ml of
the B.subtilis culture and poured immediately into petri
dishes (25 ml/plate). After drying the plates in a
laminar flow hood for 15 minutes, wells (0.4mm in
diameter) were cut using a cork borer and 20 microlitres
of the test compound as a solution in ethanol (5-10 mg/L)
was added to each well. The plates were kept at 4%C for

127

5-7 hours to allow the compound to diffuse, and the
plates were then incubated overnight at 30%C.   Clear
zones of growth inhibition were seen with both (i-but)-
and (s-pent)- erythromycin A.

Although the present invention is illustrated by the
examples listed above, they should not be regarded as
limiting the scope of the invention.   The above
descriptions illustrate first, how a specific promoter
for a Type II PKS gene set, coupled to its specific
cognate activator gene, contrary to expectation, may be
used to achieve controlled and enhanced expression of
Type 1 PKS genes in a heterologous host.   Examples of
these hosts that are given are S. erythraea and S.
avermitilis, but it will.be evident to those skilled in
the art that alternative hosts, drawn from a wide range
of actinomycetes, will equally well serve as expression
hosts.   Similarly, although the actI promoter and its
cognate activator gene actII-orf4 have been used in these
Examples, it will be evident to those skilled in the art
that other Type II PKS promoter/activator gene
combinations are well-known and characterised which will
be equally efficacious in directing the controlled and
enhanced expression of Type 1 PKS genes in heterologous
cells drawn from a wide range of actinomycetes.   Examples
of such promoter/activator gene combinations include the
promoters of the dnr gene cluster and the dnrI activator
gene from the daunorubicin gene cluster of Streptomyces
peucetius: (Madduri, K. and Hutchinson, C.R. J. Bacteriol
(1995) 177:1208-1215) and the promoter of the gene redX
and the activator gene redD from the undecylprodigiosin
gene cluster of S. coelicolor (Takano, E. et al. Mol.
Microbiol. (1992) 2: 2797-2804).

Secondly, the above descriptions illustrate for the first
time the construction of hybrid Type I PKS genes and
their use to obtain novel polyketide products of utility

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as chiral synthetic intermediates or as bioactive
materials such as antibiotics. Hybrid PKS genes have
been constructed either by substitution of loading
modules, or by substitution of individual domains in
extension modules; or by substitution of whole modules.
Thus, the replacement of the ery loading module by the
avr loading loading module has been described herein to
obtain either novel erythromycin A analogues or triketide
lactones. It will readily occur to those skilled in the
art that other alterations of the ery loading module can
be obtained through its replacement with the loading
module of other Type I PKS gene sets. Examples of such
alterations include replacement with the loading module
of the rap PKS; and with the loading module of the FK506-
producing PKS. Such alterations will lead to the
synthesis of polyketides specifically altered in their
starter unit.

It is well-known to those skilled in the art that the avr
loading module is capable of accepting a wide range of
non-natural carboxylic acids as alternative starter
units, when these are included in the fermentation
medium. Therefore in the light of the present invention,
it is evident that in addition to the synthesis of novel
erythromycin A derivatives in which the C-13 substituent
is isopropyl or sec-butyl instead of ethyl, which has
been shown here, many other novel erythromycin A
derivatives can be obtained by feeding of the appropriate
non-natural carboxylic acids (or compounds convertible to
them by fermentation) to an appropriate strain housing
the hybrid PKS, such non-natural carboxylic acids having
in general the formula R-COOH, where R is an alpha-
branched group, and where the carbon bearing the -COOH
group is also attached to at least two other atoms or
groups other than hydrogen, with the preferred non-
natural carboxylic acids being those described for the
production of non-natural avermectins in European Patent

EP 214,731, March 18 1987, Pfizer). The resulting novel
analogues of erythromycin A can be converted, by
procedures well understood in the art, into further novel
semi-synthetic derivatives of erythromycin A, of
considerable utility in the treatment of bacterial
infection, including for example ketolides and azalides.
These embodiments of the invention are novel chiral
materials of potential utility in the chemical synthesis
of valuable bioactive products. The products which are
14-membered macrolides are novel erythromycin A analogues
which are highly valuable antibacterial agents having the
same microbial targets as do the known erythromycins and
the semi-synthetic derivatives of known erythromycins,
such as the ketolides disclosed in French patents Nos.
2697523 (06/05/94) Roussel Uclaf; 269724 (06/05/94)
Roussel Uclaf; and 2702480 (16/09/94) Roussel Uclaf.

It will be evident to those skilled in the art that the
replacement of the ery loading module by the loading
module of the rap PKS will also lead to novel and useful
analogues of erythromycin A, in which the natural
propionate starter unit is substituted by a
cycloalkylcarboxylic acid starter unit. Further examples
of the formation of such hybrid Type I PKS include, but
are not limited to, the replacement of the rap loading
module in Streptomyces hygroscopicus by the avr loading
module, leading to the formation of non-natural
rapamycins; and the replacement of the avr loading
module in Streptomyces avermitilis by the rap loading
module, leading to the formation of further examples of
non-natural avermectins. The present invention also
encompasses mutants in which more than one of the genetic
manipulations described in the examples are combined.

In the light of the present invention, it will also be
evident that alterations in the specificity of the
loading module of a Type I PKS can alternatively be

achieved by the mutation of the genes encoding the
natural loading module, and then selection for the
desired altered specificity, as practised for example in
the technique of in vitro gene shuffling (Stemmer, W. P.
Nature (1994) 370:389-391).

The examples listed above also teach the construction and
use of a low copy number plasmid vector pCJR101 as a
vector for delivery of PKS genes into suitable
actinomycete hosts. Plasmid pCJR101 is derived from the
plasmid SCP2* (Bibb, M. J. and Hopwood, D. A. J. Gen.
Microbiol. (1977) 154:155-166) found in the strain
Streptomyces coelicolor M110 deposited for example at the
Northern Regional Research Laboratory, Peoria, Illinois,
USA under the accession number NRRL 15041. Plasmid SCP2*
has been previously used in the construction of several
useful vectors such as pIJ2839 (Ingram, C. et al. J.
Bacteriol. (1989) 171:6617-6624); plasmid pHJL197
(Larson, J. L. and Hershberger, C. L. J. Bacteriol.
(1983) 157:314-317) and pRM5 (McDaniel, R. et al. Science
(1993) 262:1546-1550). It will be evident to those
skilled in the art that either these or other SCP2*-based
plasmids may be substituted for pCJR101 either directly
or after modification of the vector to introduce suitable
promoter linked to the PKS genes, as demonstrated by the
use of plasmid pRM5 in several Examples described herein.
High copy number vectors such as plasmid pGM8 (Muth, G.
et al. Mol. Gen. Genet. (1989) 219:341-350) derived from
the Streptomyces ghanaensis plasmid pGS5 are also
suitable as substitutes for pCJR101, as are integrative
vectors such as plasmid pSAM2 (Murakami, T. et al. J.
Bacteriol. (1989) 171:1459-14??). Those skilled in the
art will readily appreciate the versatility of approaches
to increasing the rate of biosynthesis of natural or non-
natural complex polyketides such as macrolides and
polyethers through heterologous use of type II PKS
activator genes and their cognate promoters as disclosed

131

here, in numerous derivatives of vectors well known in
the art as useful for genetic engineering in
actinomycetes.

In the construction of hybrid type I PKS genes, the
Examples teach how the structural genes encoding both
donor and acceptor PKS components may be spliced together
to create functional catalysts capable of bringing about
the synthesis of novel polyketides. The present
invention shows that in choosing where the junction will
be made between the donor and the acceptor DNA it is,
surprisingly, not necessary to limit the choice to
positions known or predicted to lie between domains in
so-called linker regions. Instead it is preferred for
junctions to be in the edge regions of domains
(particularly KS or AT domains), where the sequences are
highly conserved. Further, creation of junctions that
lead to conservative changes in amino acid sequence at
such junctions in the gene product are tolerated. It is
also evident that for the purposes of creating a hybrid,
PKS modules may be combined from two or more natural PKS.
In the examples given here, donor DNA is spliced into the
acceptor DNA at a position variously in the acyl carrier
protein (ACP) domain or in the ketosynthase (KS) domain
of a module, but the scope of the invention includes
hybrid PKS where the junctions between homologous domains
are chosen to lie within any of the constituent parts of
a type I PKS module. However, it will be found most
advantageous to select a position for each junction that
lies within a domain, and close to one edge, so that the
specificity of the chimaeric module is readily
predictable, and so that disturbance of its proper
functioning is minimised.

It will be readily appreciated that in the light of the
present invention a hybrid PKS can be constructed by
selecting pieces of DNA encoding respectively a loading

module, a variable number of extension modules up to at least six in number, and a chain-releasing thioesterase domain; and concatenating the DNA, using standard procedures, in the order in which it is intended that the gene products operate. NB: The hybrid PKS with (say) 6 modules may be part of an assembly of synthases leading to a product produced by many more than 6 extension modules. It will also readily occur to those skilled in the art that the module-sized DNA fragments may be constituted in more than way, Thus the present invention includes the construction of functional hybrid PKSs exemplified by the construct containing the following activities in a single polypeptide chain:

AT0-ACP0-KS1-[ATR1-DHR1-ERR1-KRR1-ACPR1-KSR2]-AT2-KR2-ACP2-TE

where the activities shown in square brackets are derived from modules 1 and 2 of the rap PKS, and the rest are derived from the loading module, extension modules 1 and 2, and the chain-terminating thioesterase of DEBS1. In such constructs, each ketossynthase domain is kept together with the ACP, AT and reductive domains of the module that precedes it in the naturally occurring PKS from which it was derived, rather than with the activities of its own module. Alternative but equally functional arrangements of the module-sized DNA building blocks for construction of hybrid PKS will readily occur to those skilled in the art.

CLAIMS

1.   A hybrid polyketide synthase ("PKS") gene comprising
a first nucleic acid portion or portions encoding at
5   least one domain of a first type I PKS and a second
nucleic acid portion or portions encoding at least one
type I PKS domain which is heterologous to said first
PKS.

10   2.   A hybrid PKS gene according to claim 1 wherein said
first nucleic acid portion encodes at least a loading
module and said second nucleic acid portion encodes at
least one extension module.

15   3.   A hybrid PKS gene according to claim 2 wherein said
loading module comprises an acyltransferase and an acyl
carrier protein.

4.   A hybrid PKS gene according to claim 2 or claim 3
20   wherein a said first nucleic acid portion encodes a
loading module together with the ketosynthase ("KS")
domain (only) of the homologous extender module.

5.   A hybrid PKS gene according to claims 2, 3 or 4
25   wherein said loading module is capable of loading a
substrate to produce a starter unit different from a
starter unit normally associated with said extension
module(s).

30   6.   A hybrid PKS gene according to any of claims 2-5
wherein said loading module is capable of loading any of
a multiplicity of different starter units.

7.   A hybrid PKS gene according to claim 6 wherein said
35   loading module is an _avr_ loading module.

8.   A hybrid PKS gene according to any preceding claim

SUBSTITUTE SHEET (RULE 26)

wherein said nucleic acid portions encode combinatorial
modules each extending between corresponding domains of
two natural modules.

5   9.   A hybrid PKS gene according to any preceding claim
       including nucleic acid encoding a chain terminating
       enzyme other than thioesterase.

       10.   A hybrid PKS gene according to any preceding claim
10   wherein said second nucleic acid portion or portions
       comprises a portion encoding an extension module leading
       to a ketide unit differing from the natural unit in
       oxidation state and/or stereochemistry and/or
       substitution pattern.

15
       11.   Nucleic acid encoding a gene according to any of
       claims 1-10 operably linked to a PKS type II promoter.

       12.   Nucleic acid according to claim 11 wherein the
20   promoter is accompanied by its natural activator gene.

       13.   Nucleic acid according to claim 11 or 12 wherein the
       promoter is act I of S.coelicolor.

25   14.   A hybrid polyketide synthase as encoded by a gene
       according to any of claims 1-10.

       15.   A vector including a gene or nucleic acid according
       to any of claims 1-13.
30
       16.   A transformed organism containing a gene or nucleic
       acid according to any of claims 1-13 and able to express
       a polyketide synthase encoded thereby.

35   17.   A method of producing an organism as defined in
       claim 16 comprising the step of introducing a plasmid
       containing 'donor' DNA into a host cell under conditions

135

such that there is homologous recombination with
heterologous chromosomal PKS DNA.

18.   A method of making a polyketide by culturing the
5   organism of claim 16.

19.   A polyketide as prepared by the method of claim 18.

20.   Use of a type II PKS promoter to control a
10   heterologous gene.

21.   Nucleic acid comprising a type II PKS promoter
operably linked to a heterologous gene.

15   22.   The use or nucleic acid according to claim 20 or
claim 21 wherein the promoter is accompanied by its
natural activator gene.

23.   The use or nucleic acid according to claim 20, 21 or
20   22 wherein the promoter is act I of S.coelicolor.

1/40
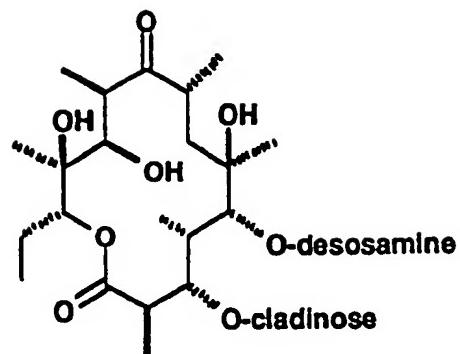
Erythromycin A
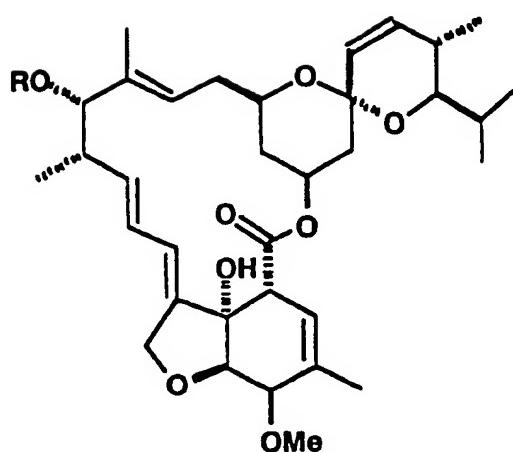
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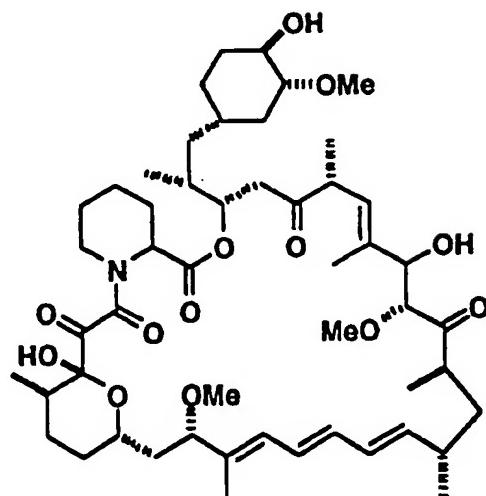
Rapamycin

Figure 1.
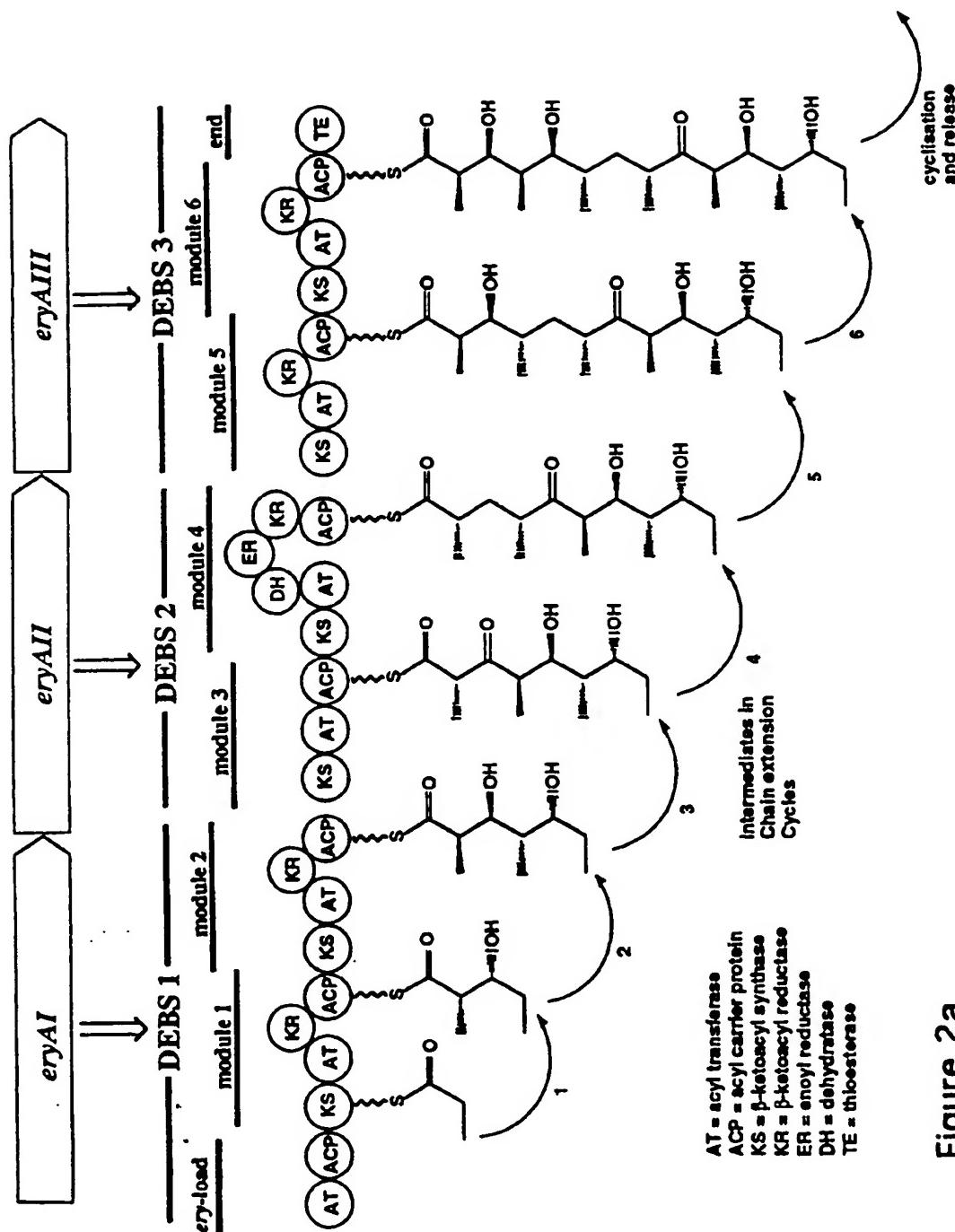
Figure 2a

3/40

Figure 2b.

4/40
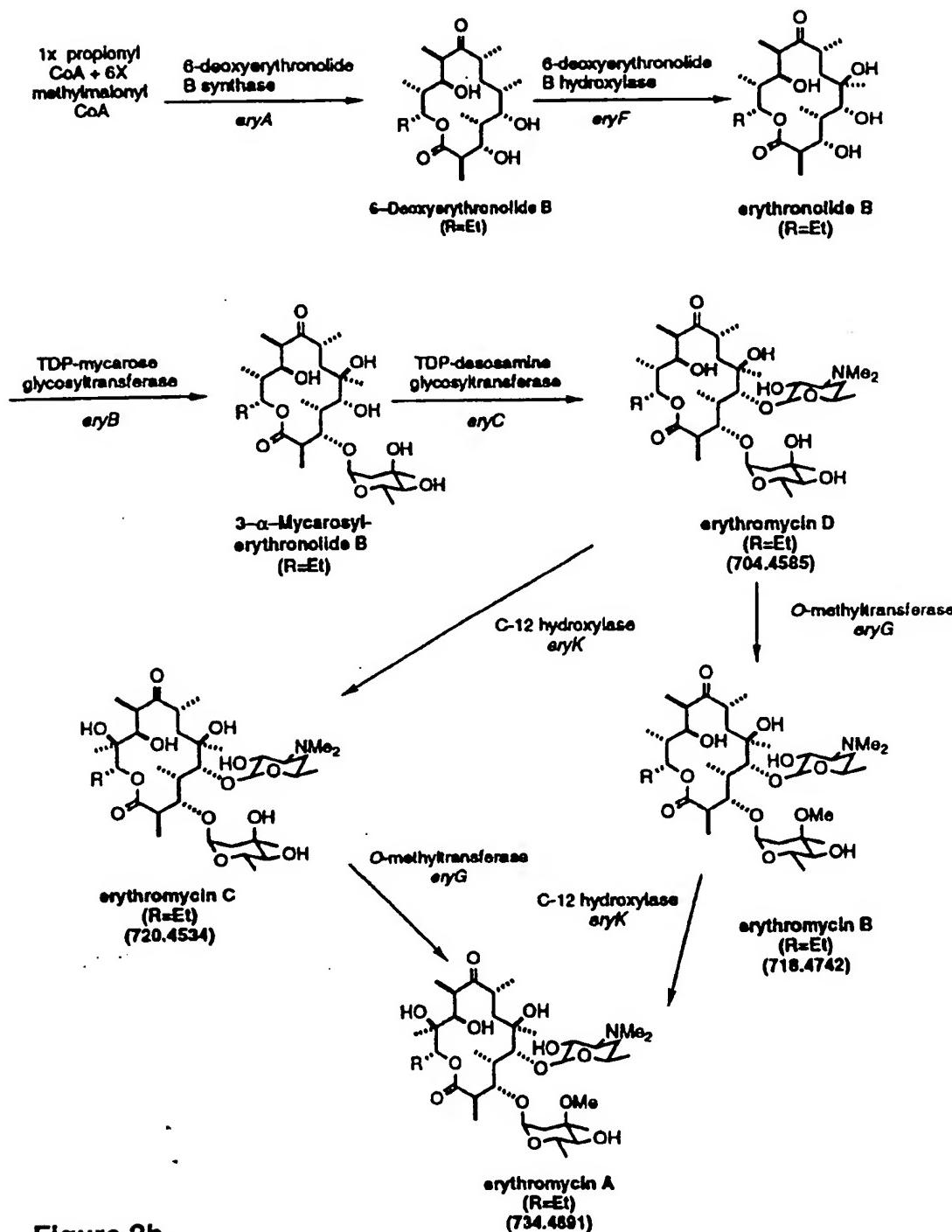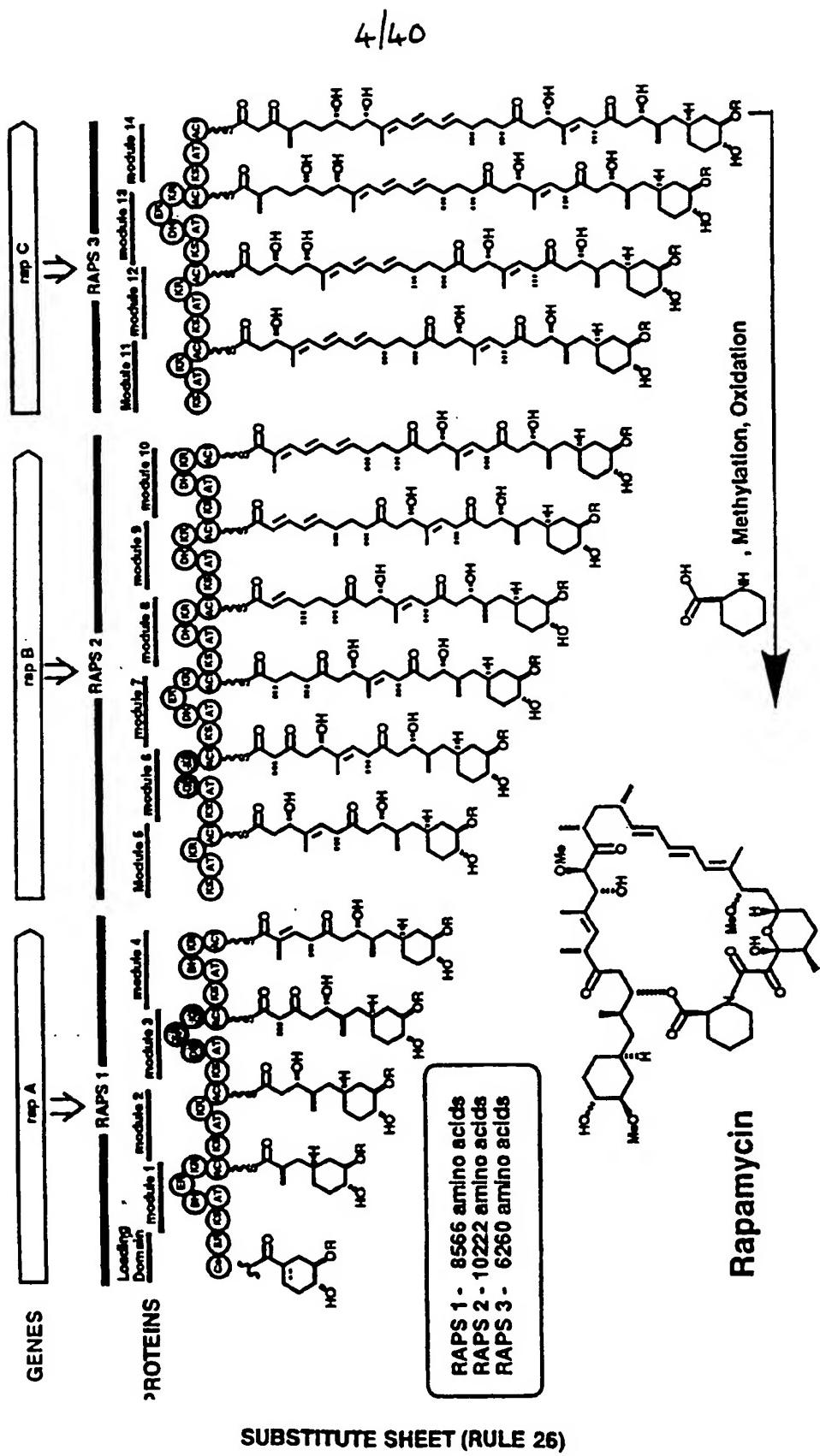
Figure 3

5/40

Figure 4

6/40
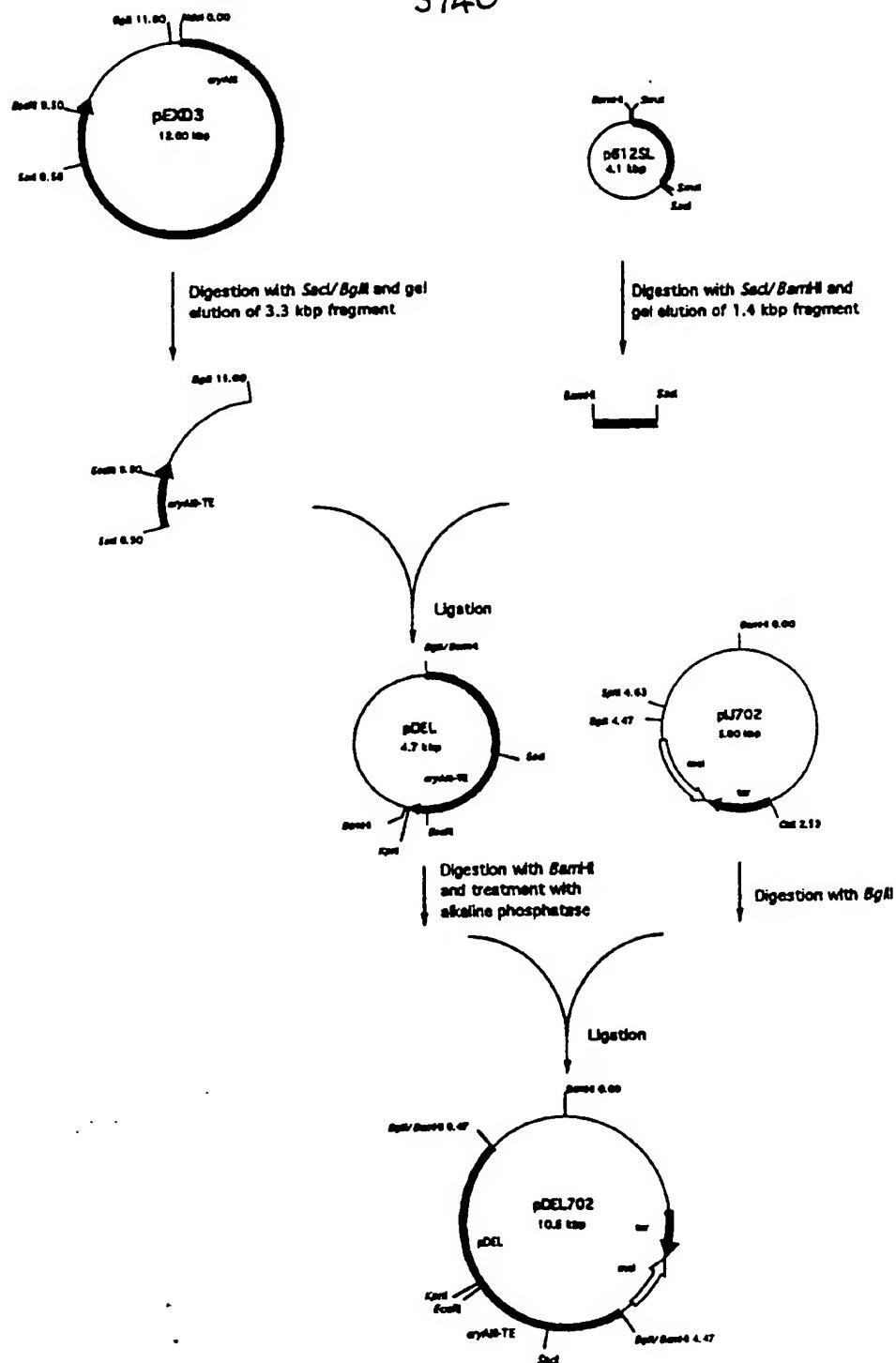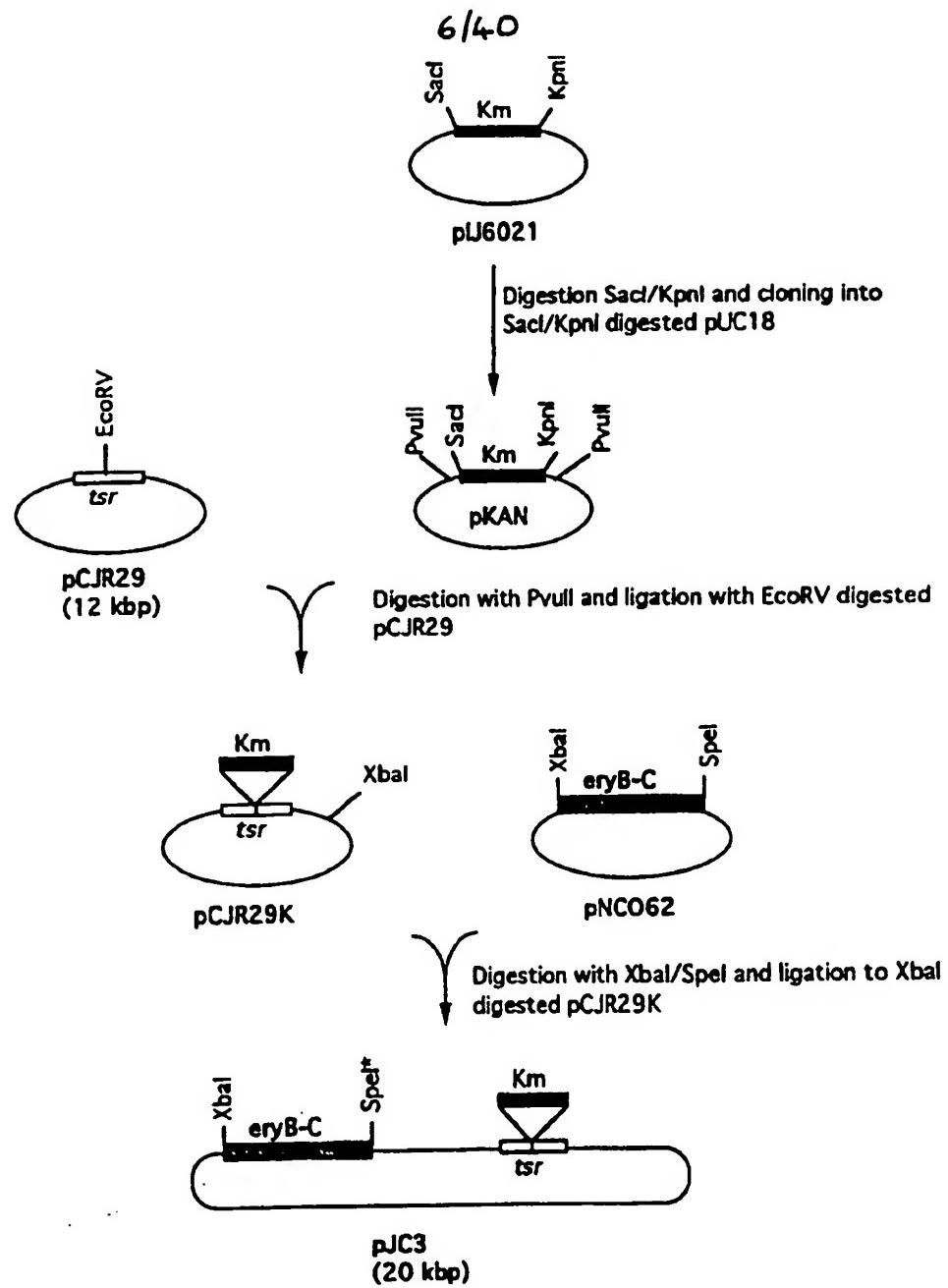
Figure 5. Construction of pJC3

7/40
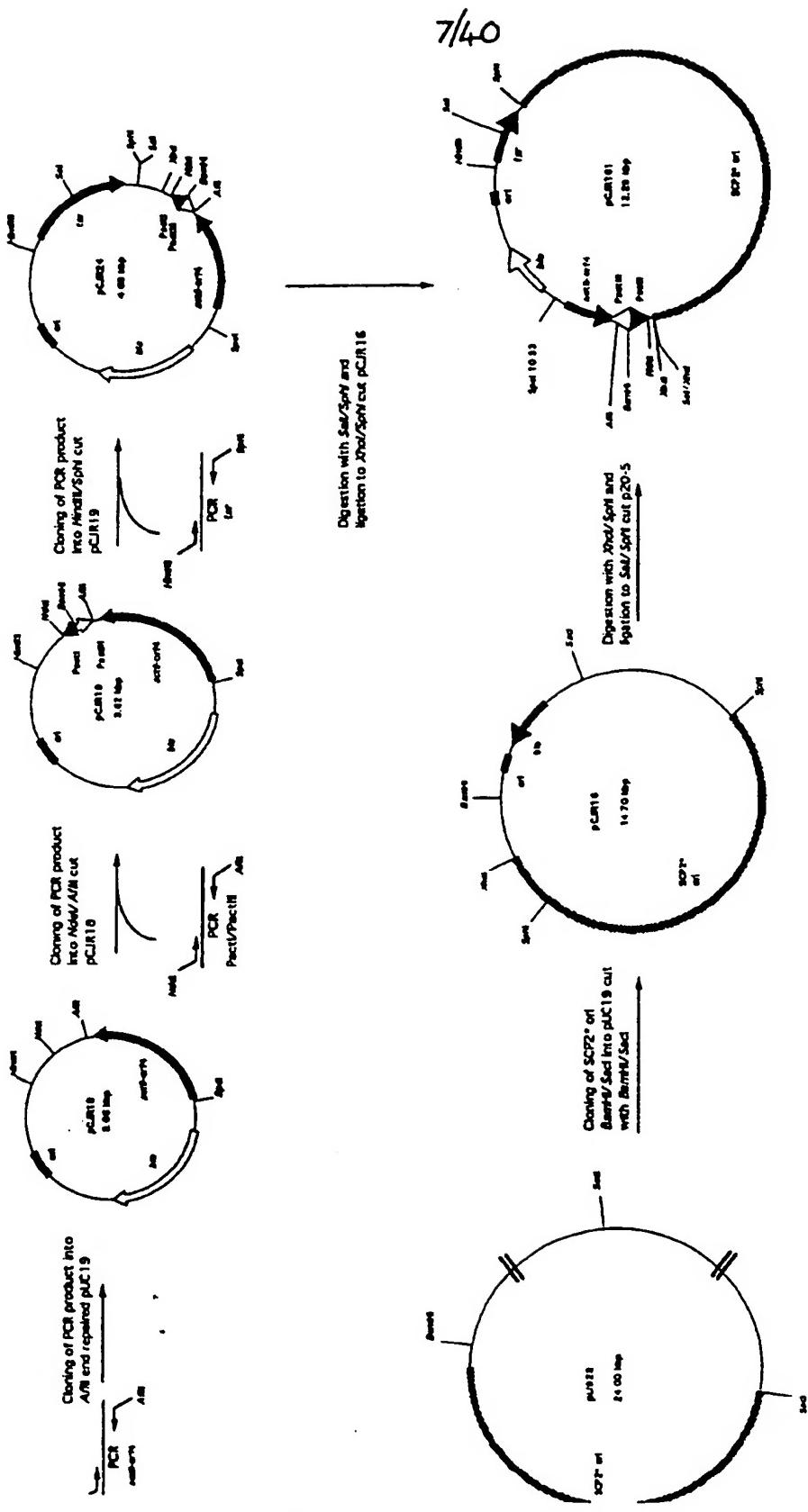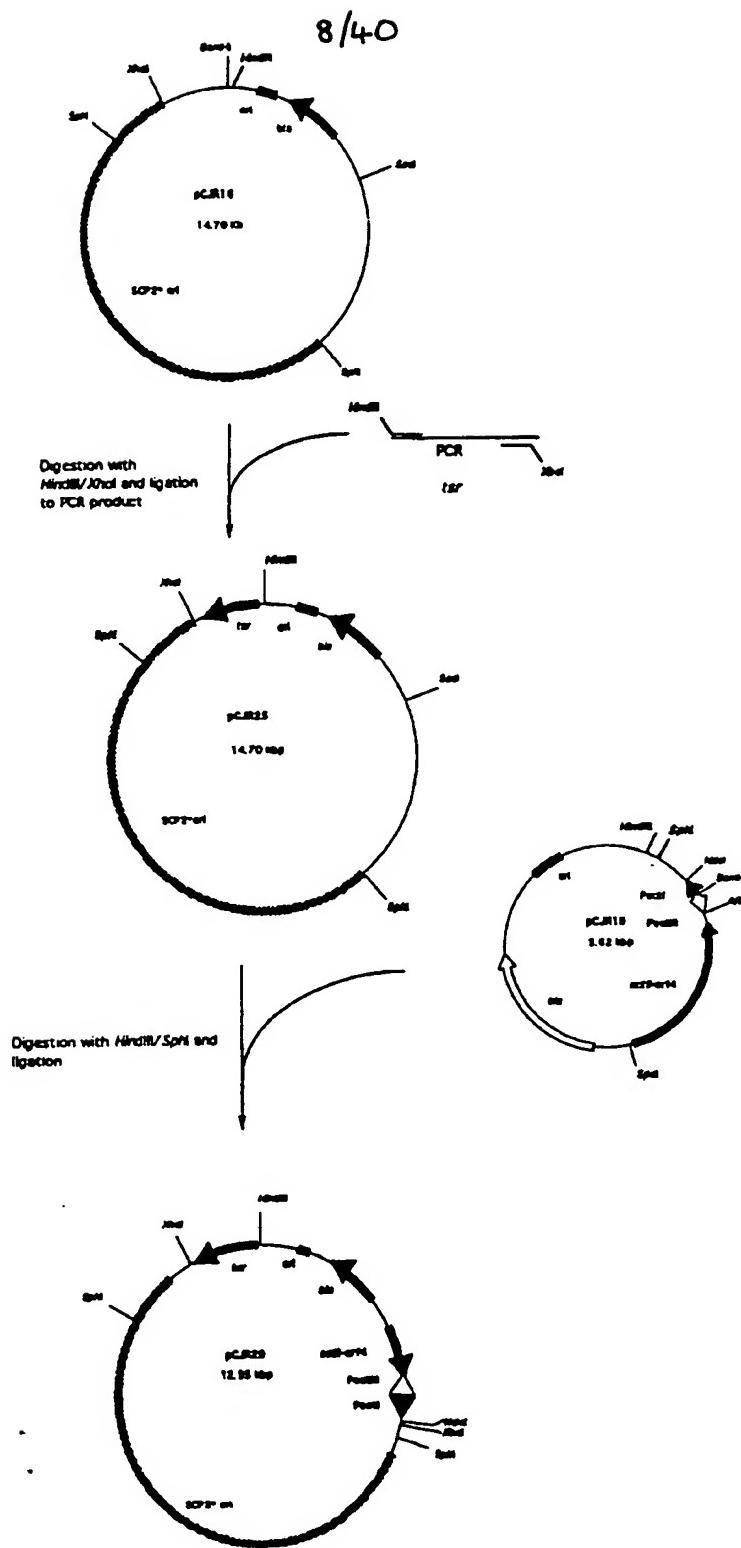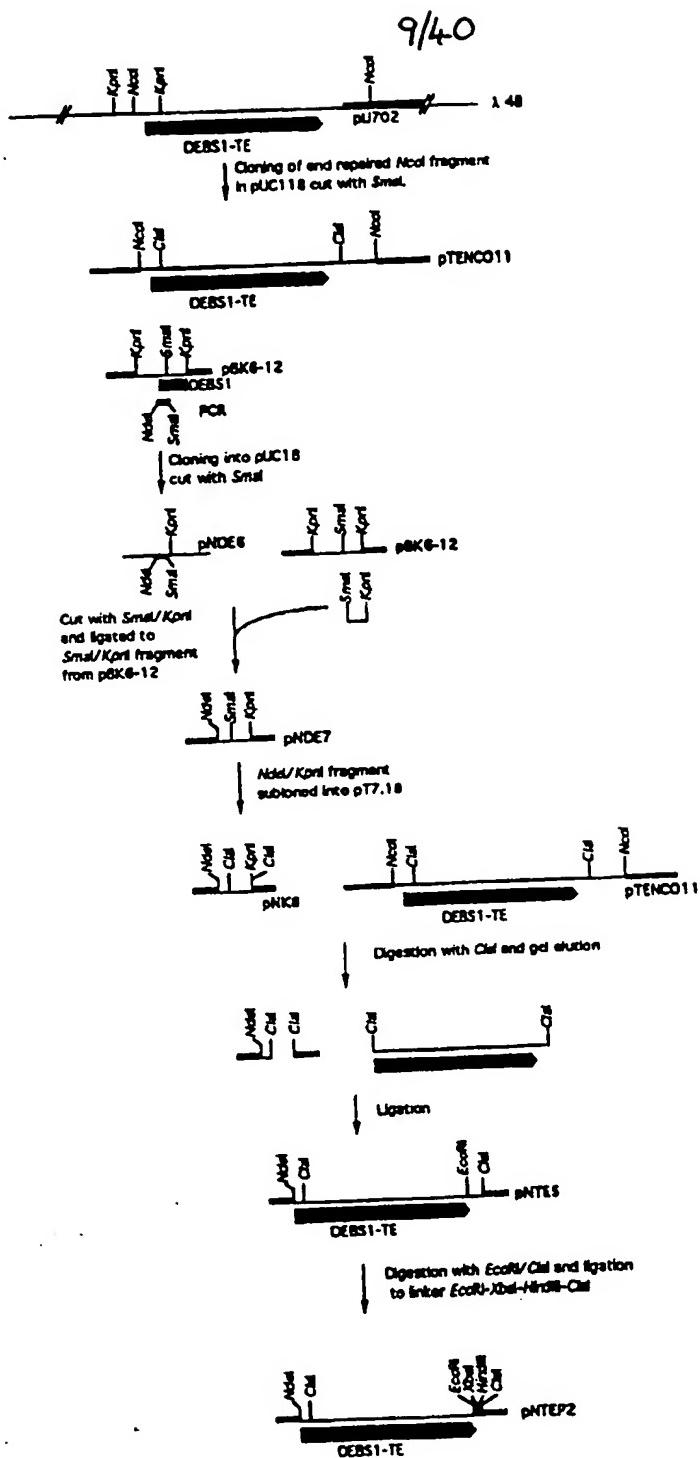
Figure 6
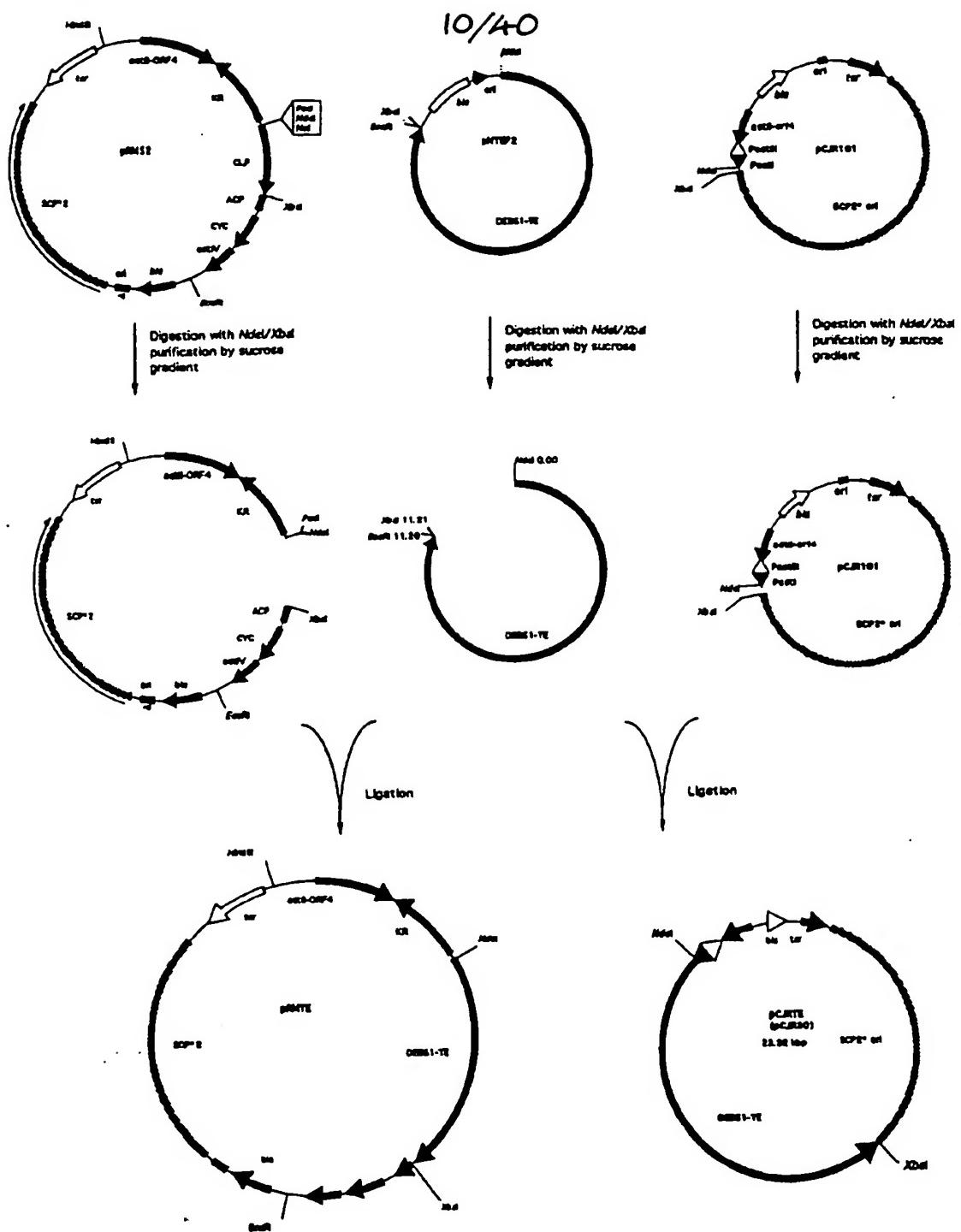
Figure 7.

Figure 8.

Figure 9.

11/40

Figure 10a.

12/40

Digestion with *NdeI/XbaI*

Ligation

Figure 10b.

13/40

figure 11

14/40

Figure 12.

15/40
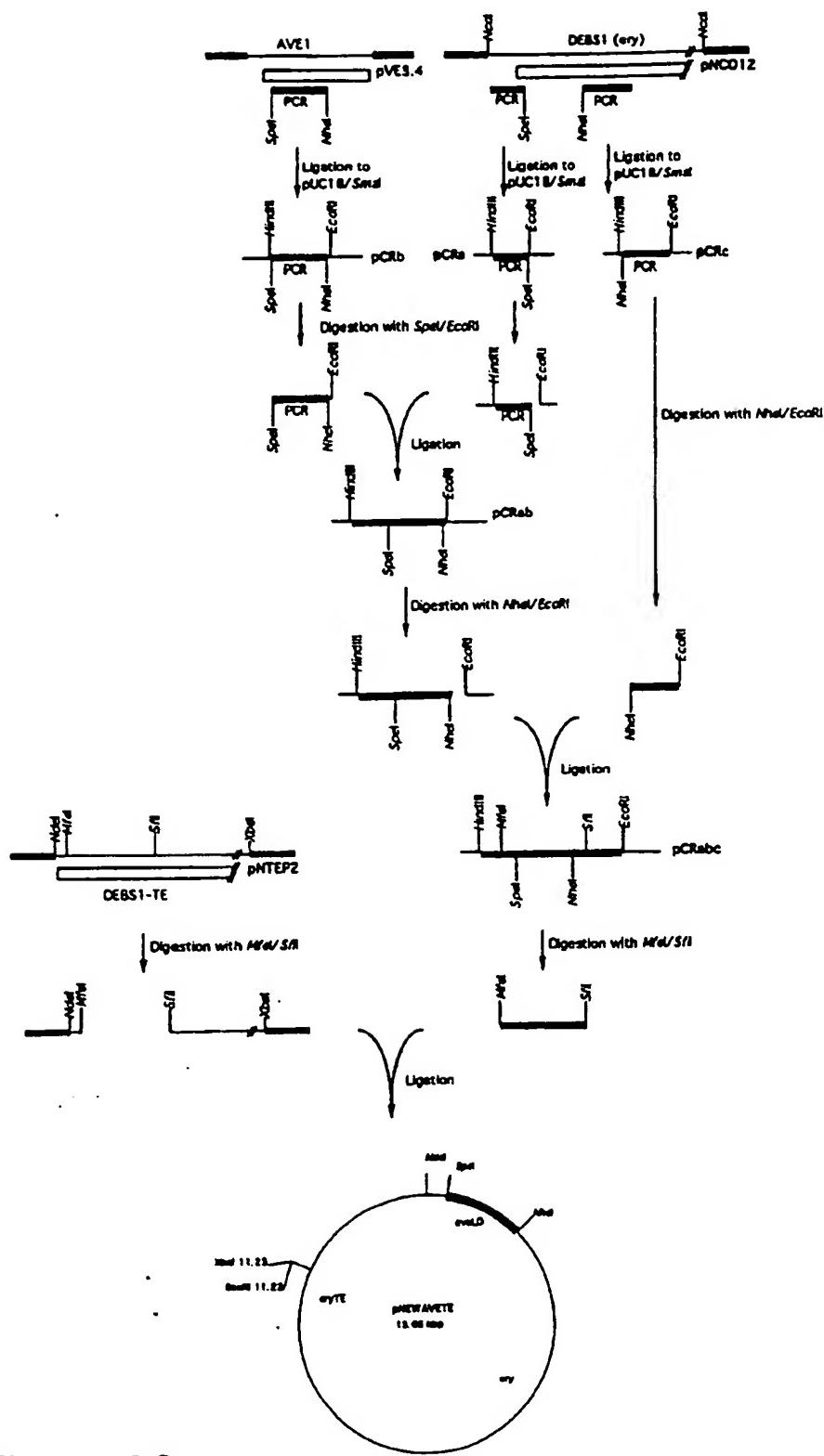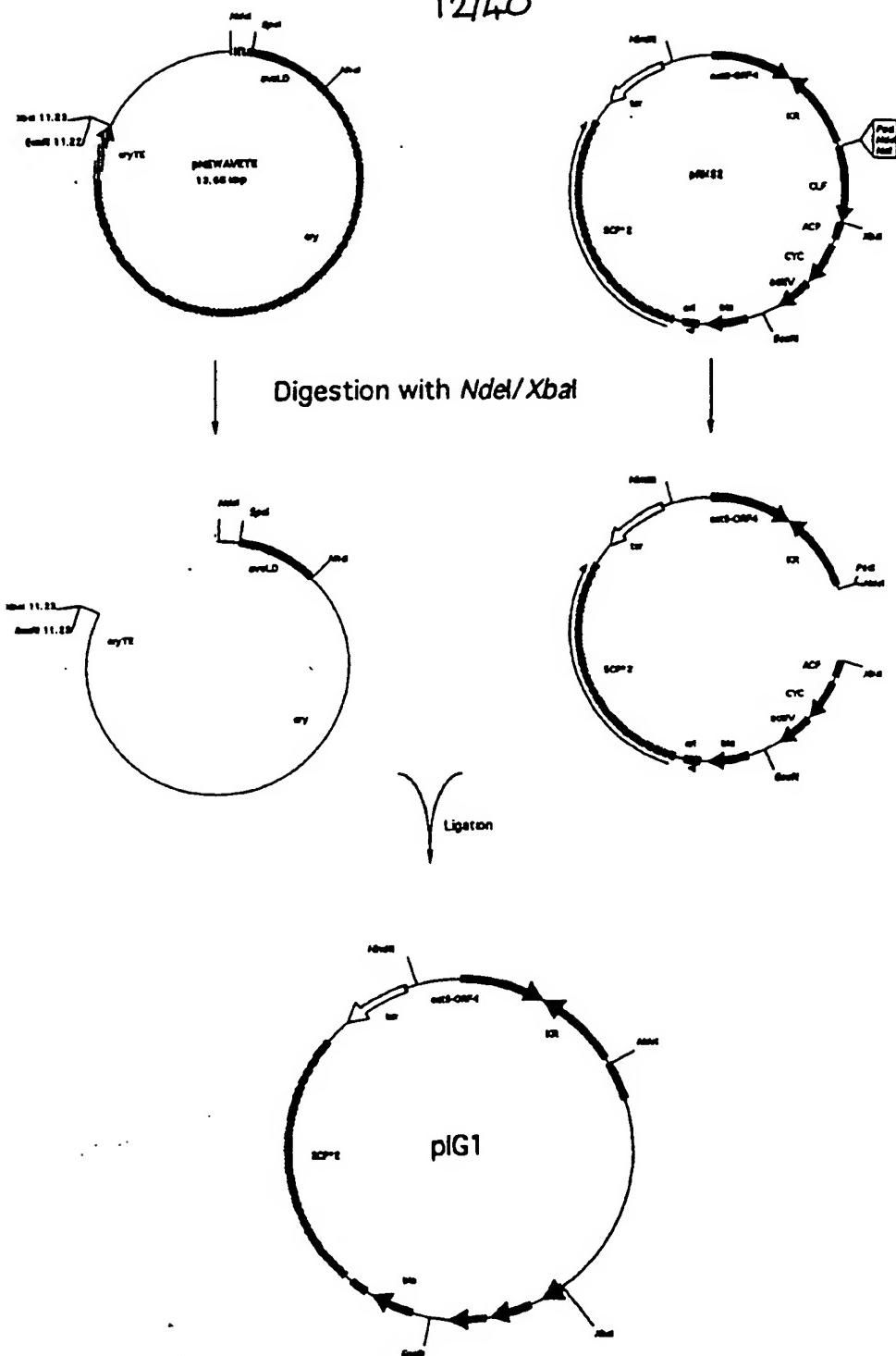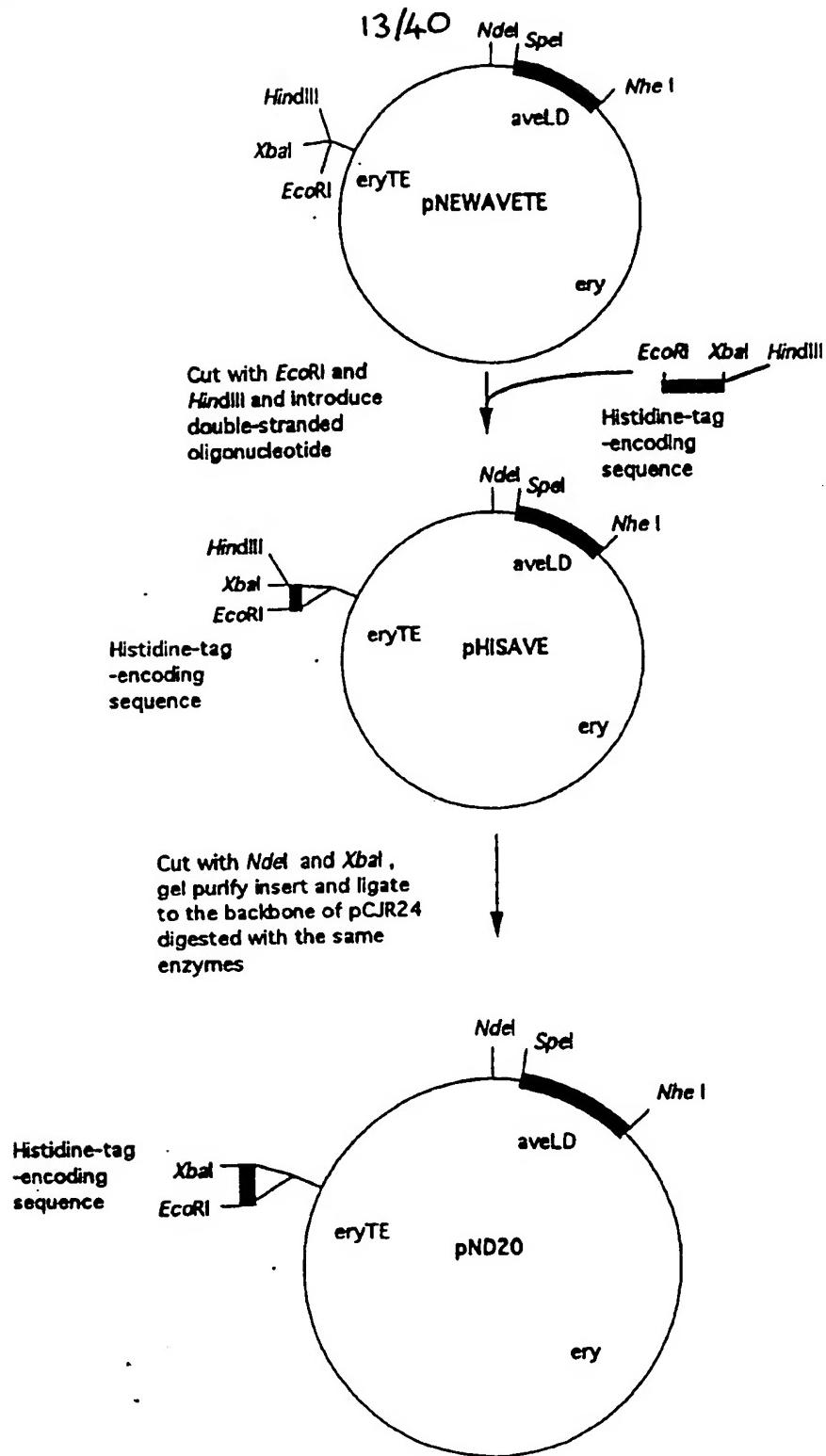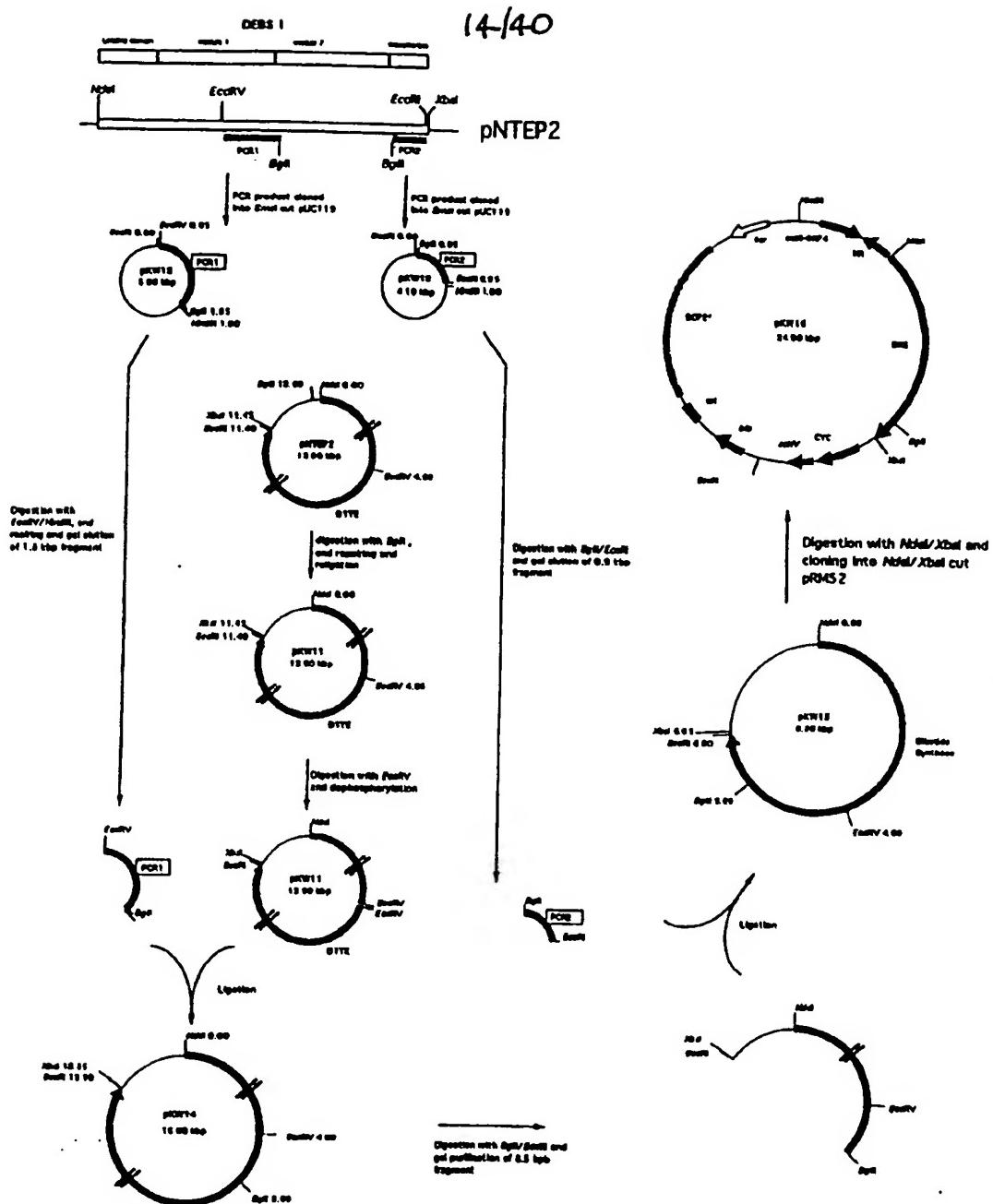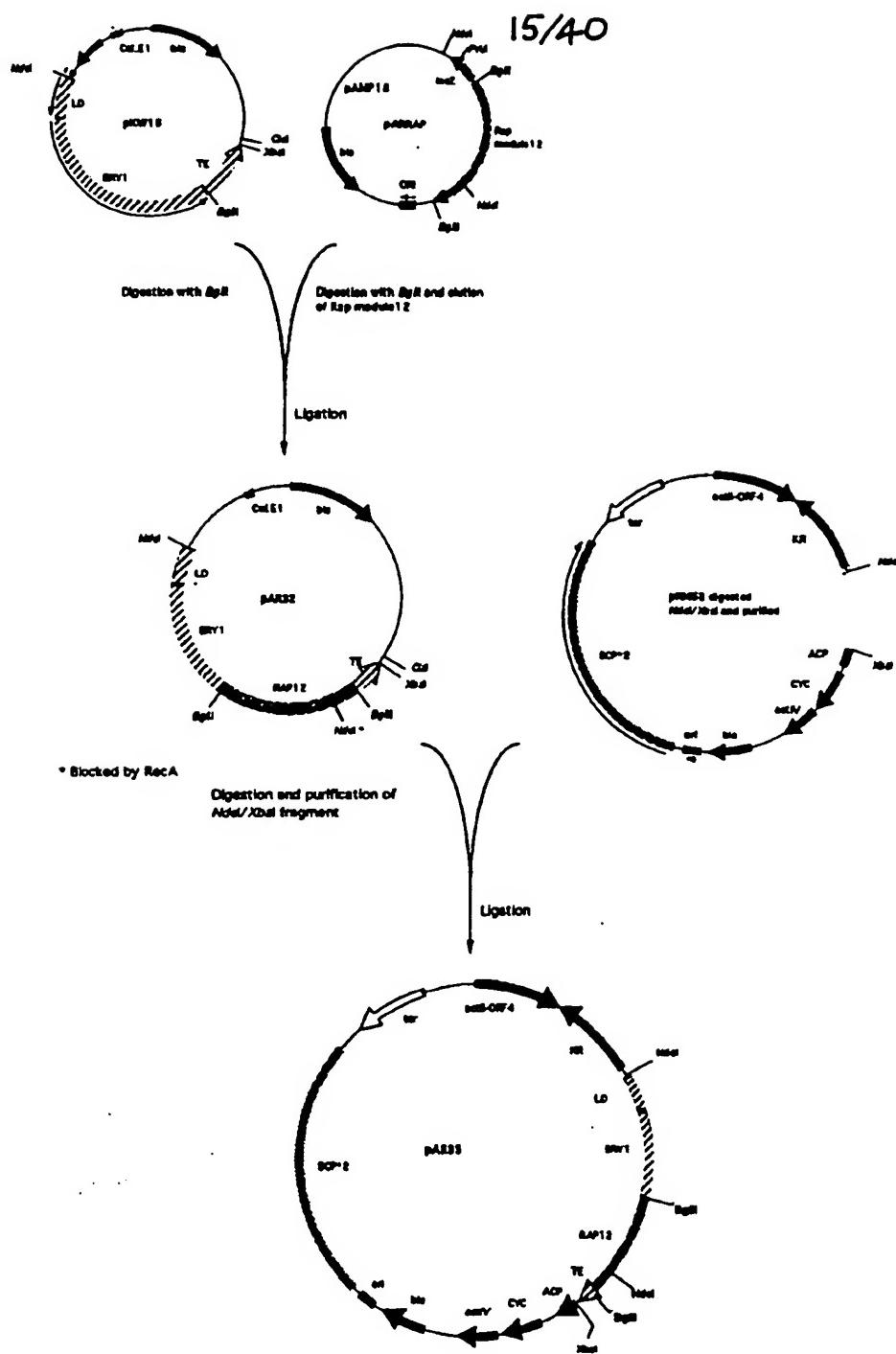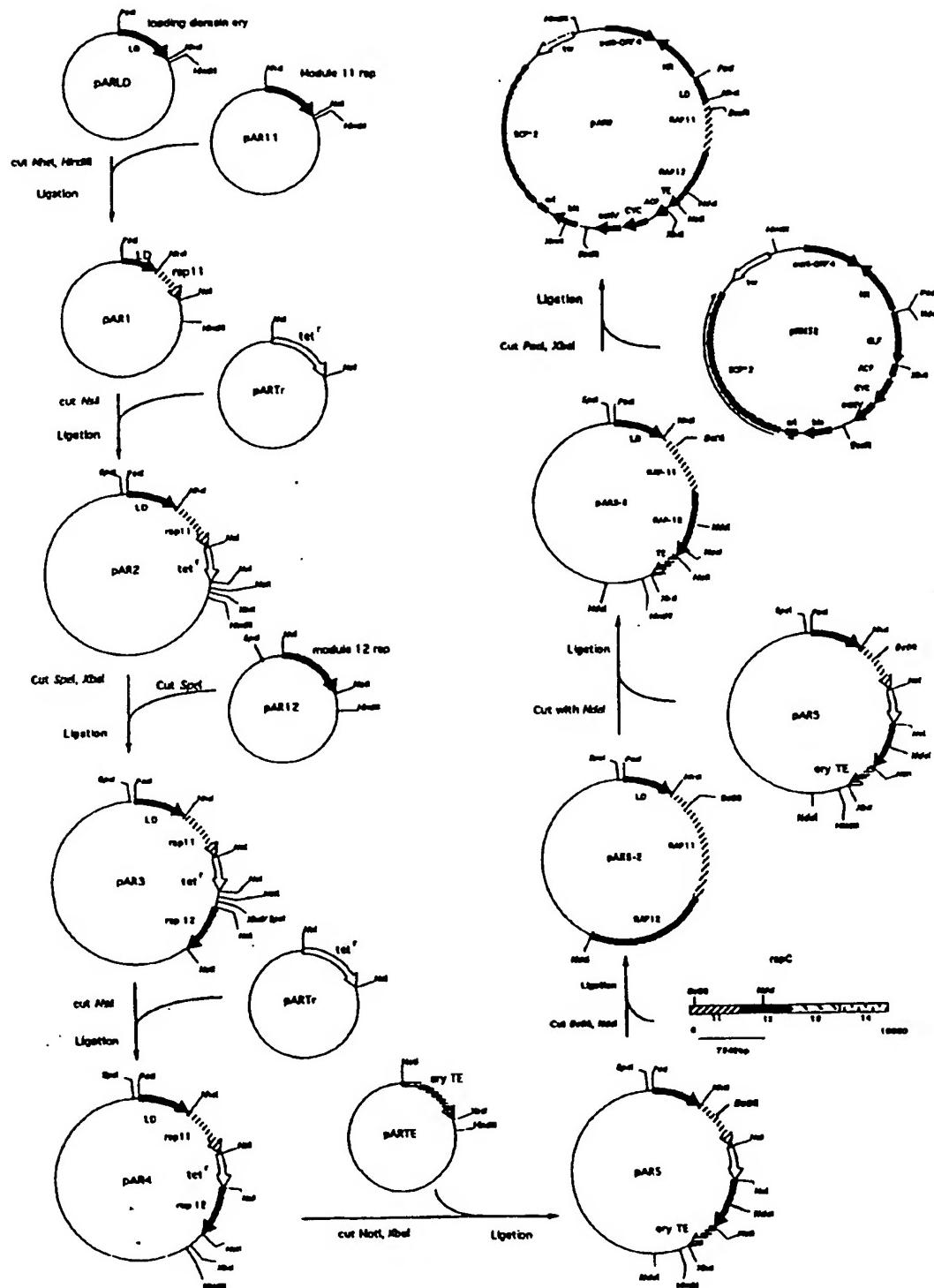
Figure 13.

Figure 14.

17/40

Figure 15.

18/40

reposition KS

native position of KS
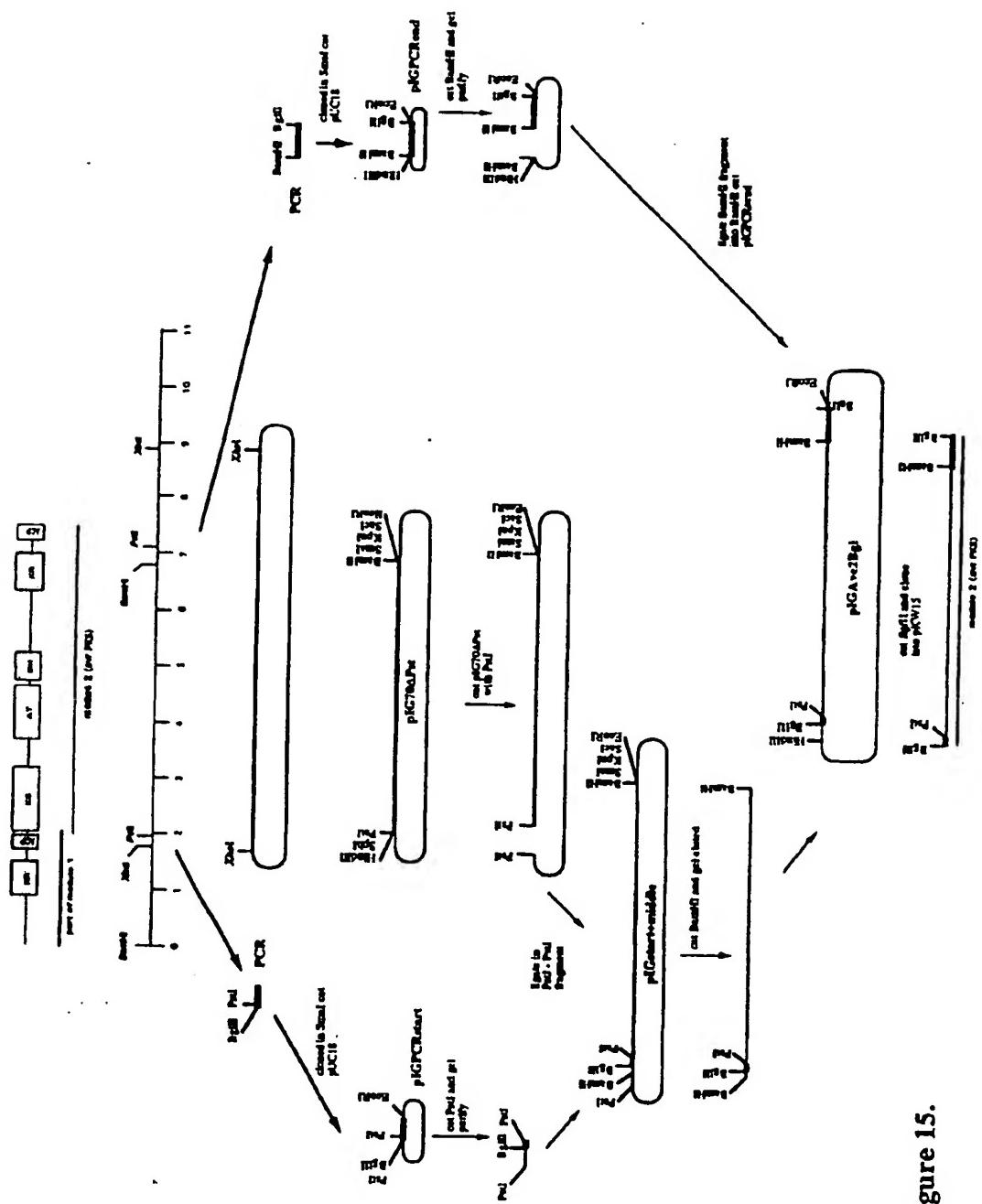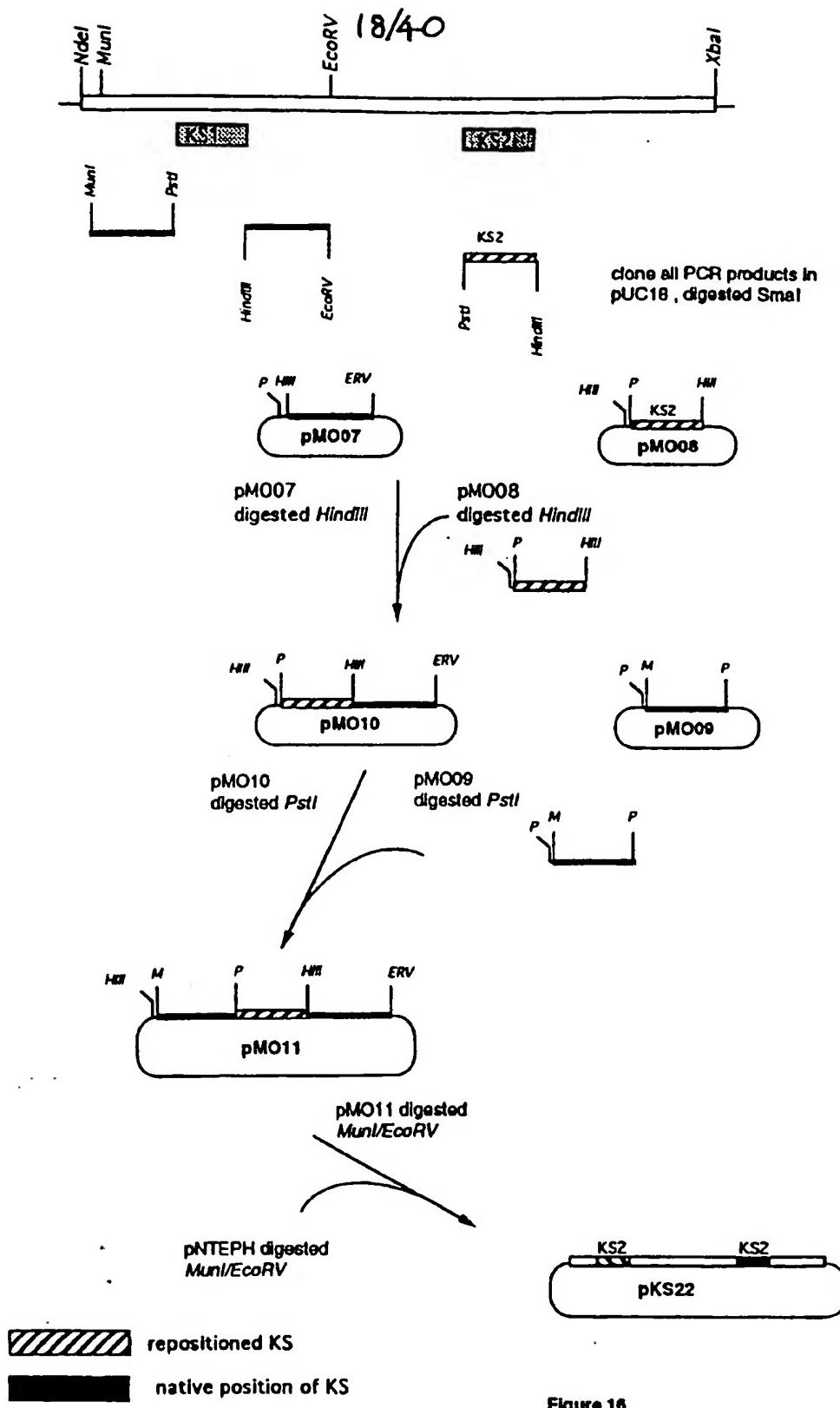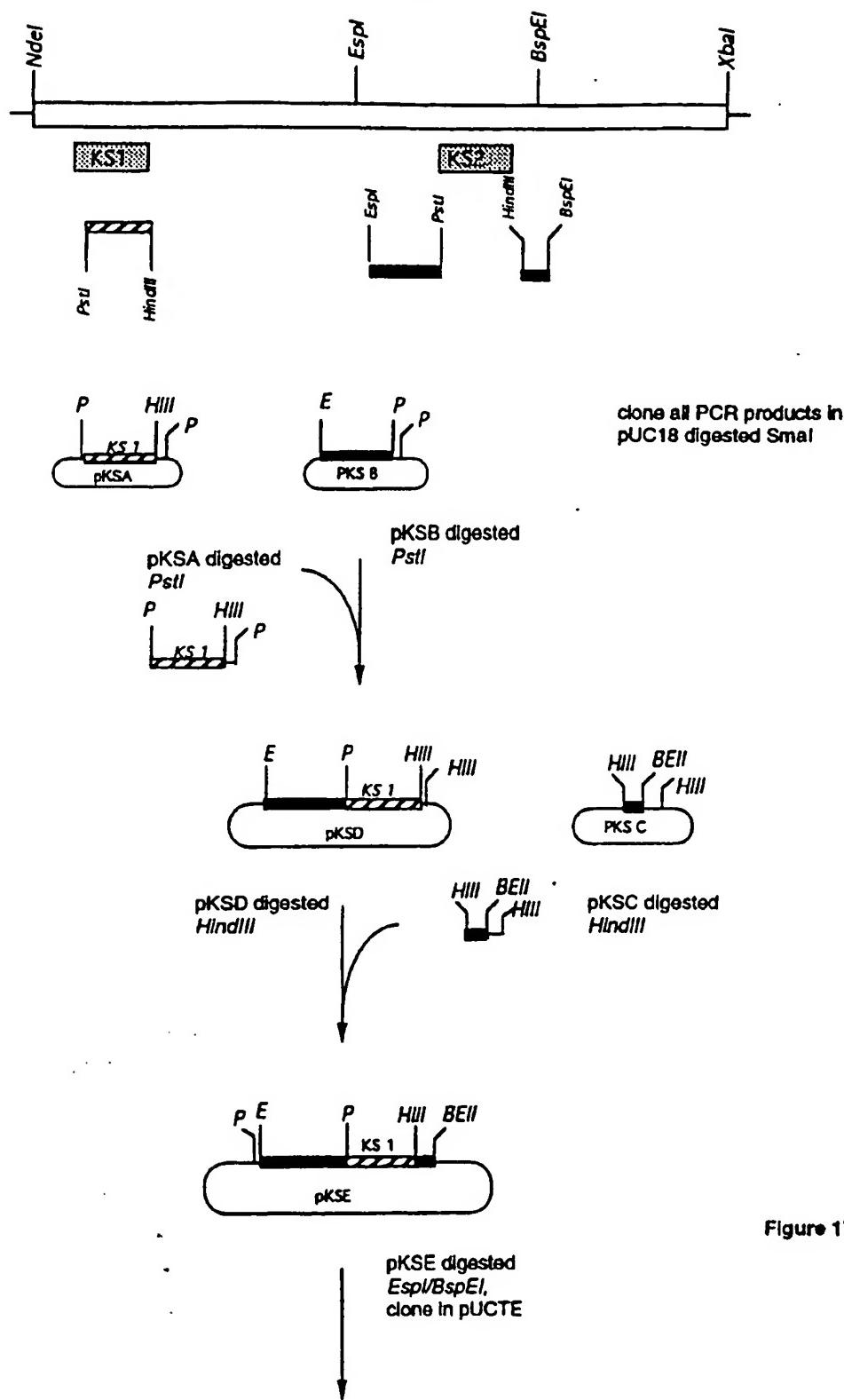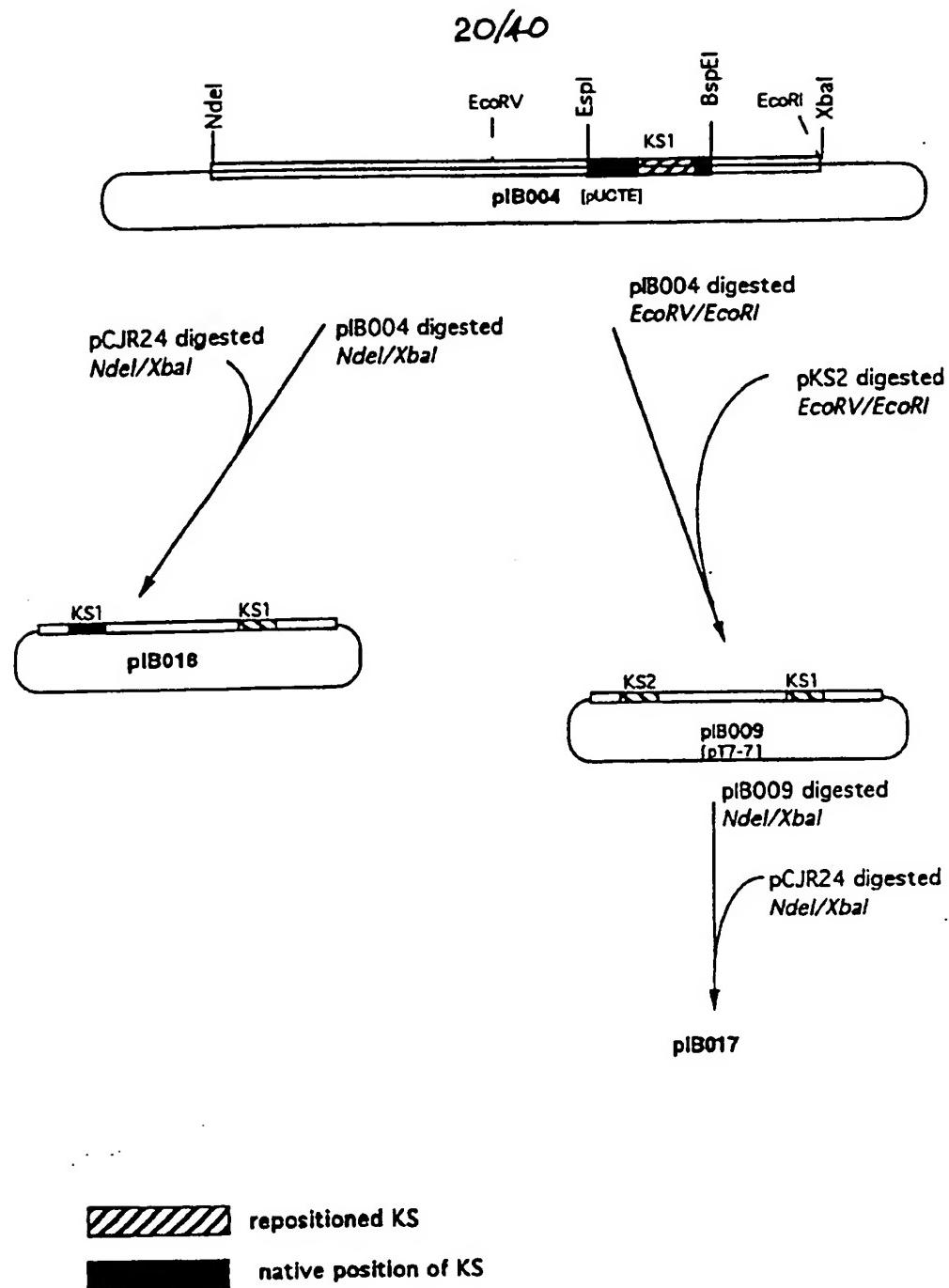
Figure 16

Figure 17a

Figure 17b
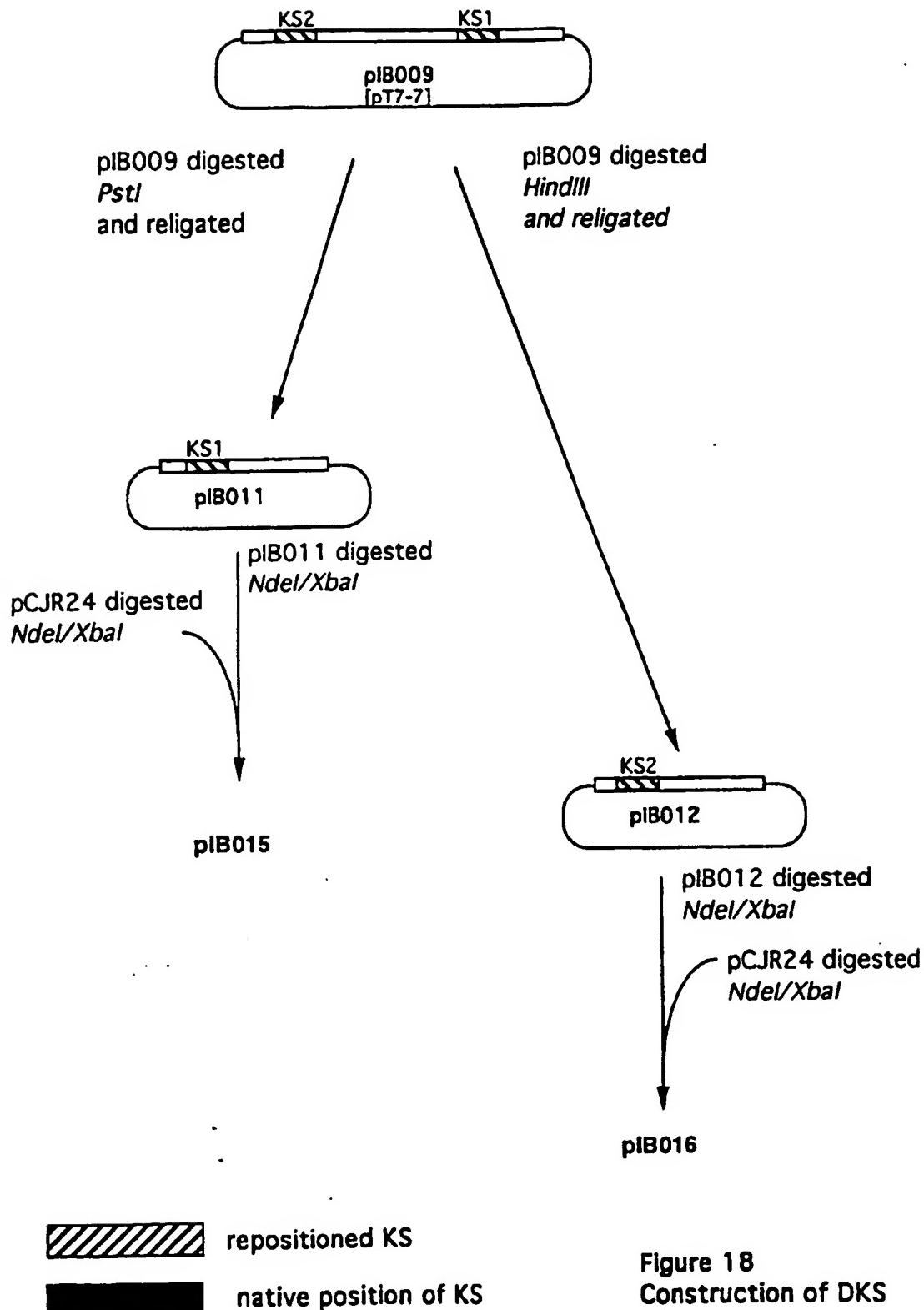
Figure 18
Construction of DKS

22/40
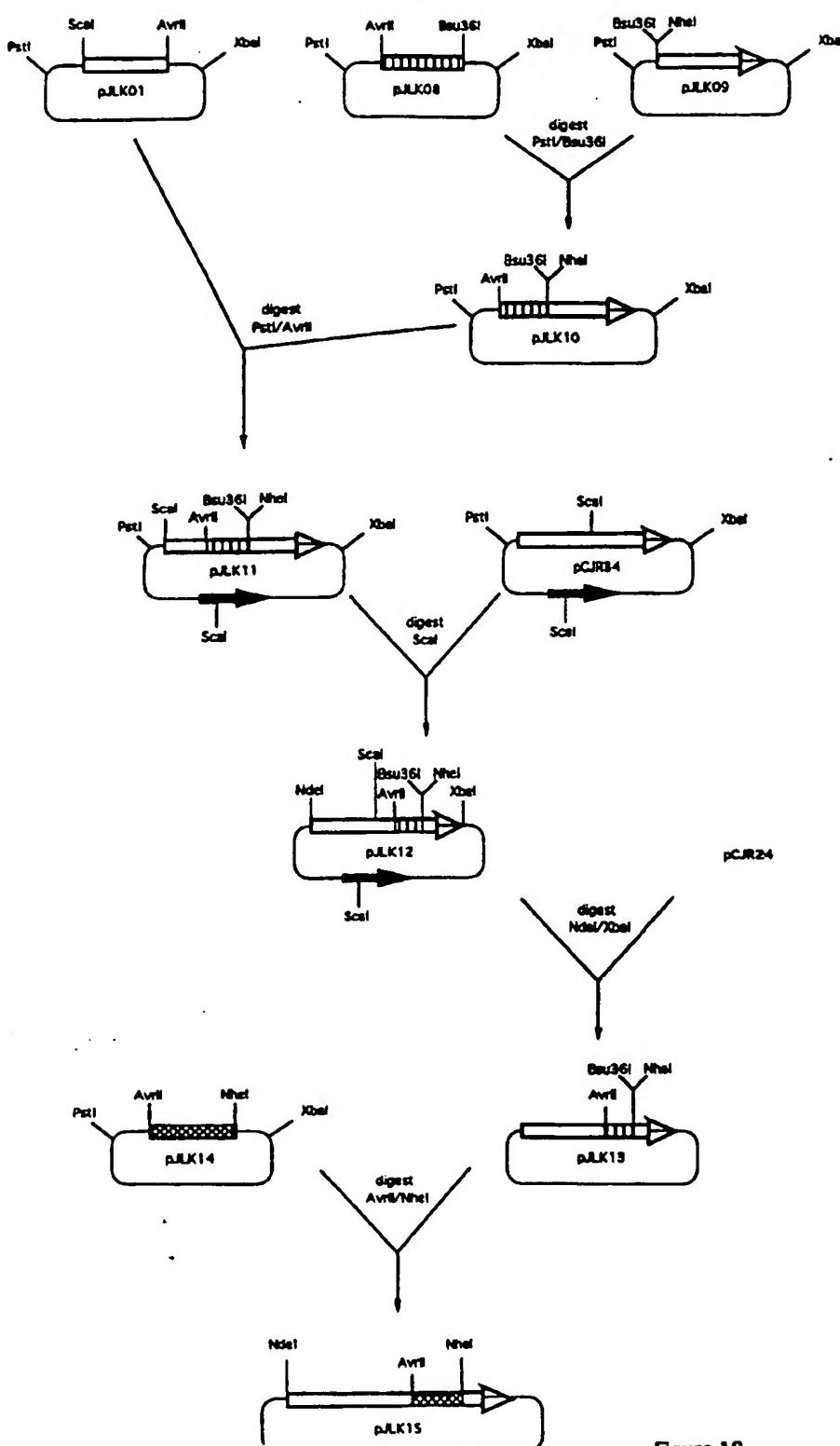
Figure 18
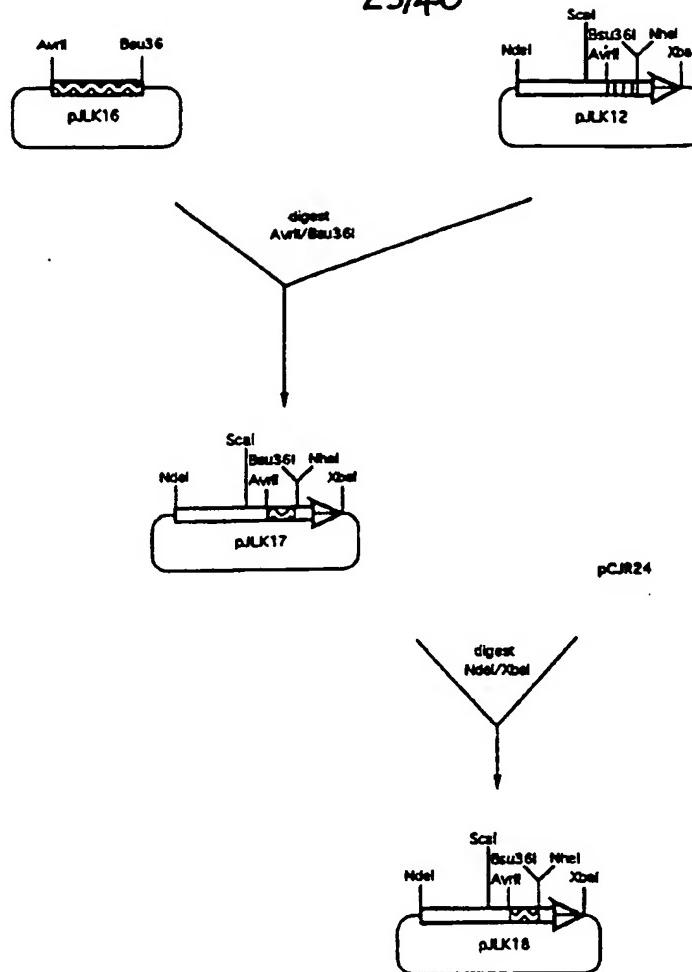
Figure 20

24/40

Figure 21

Construction of a Diketide Synthase With Different Reductive Loops

25/40

Fig 22

26/40

Fig 23. Construction of plasmid pKETO

27/40

Figure 24.

28/40
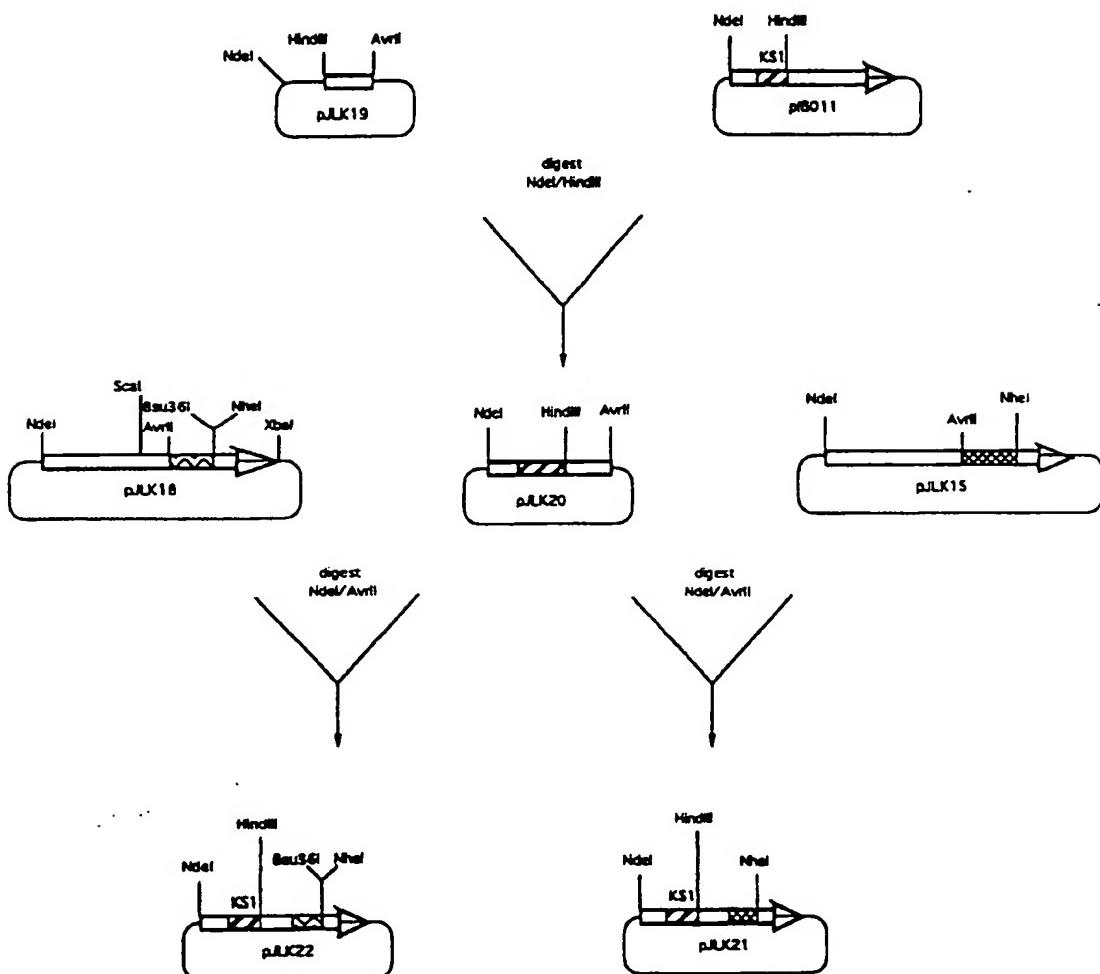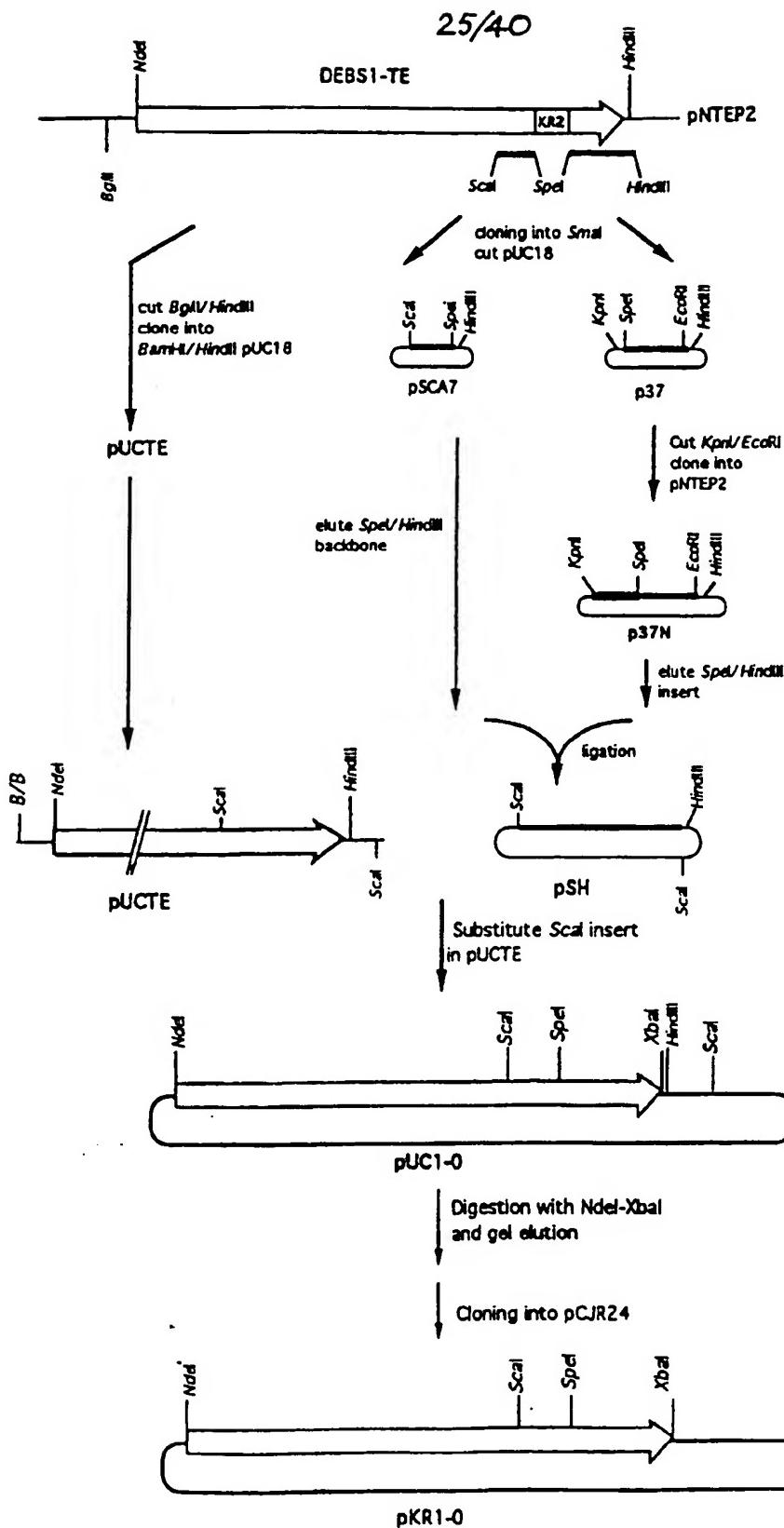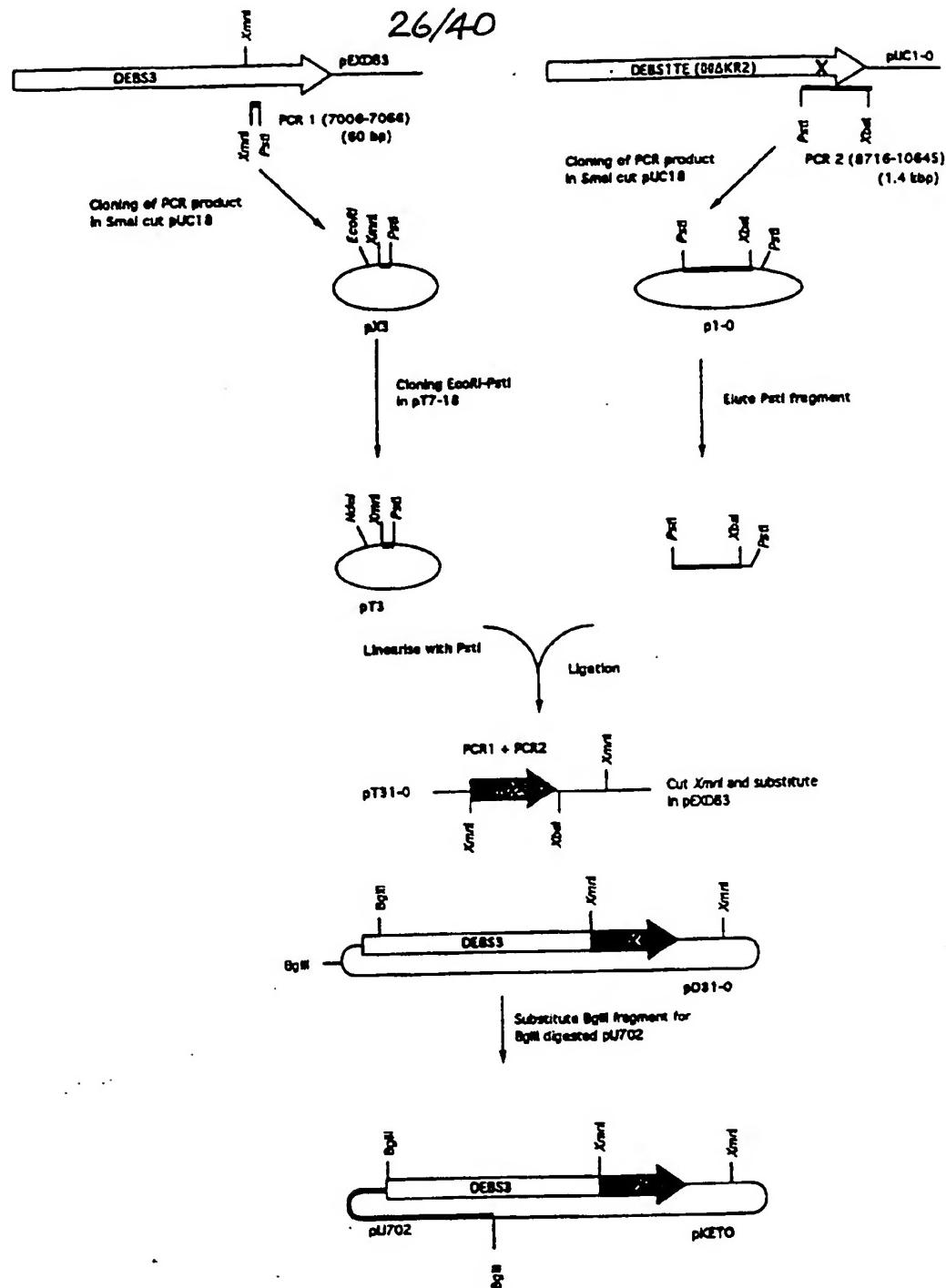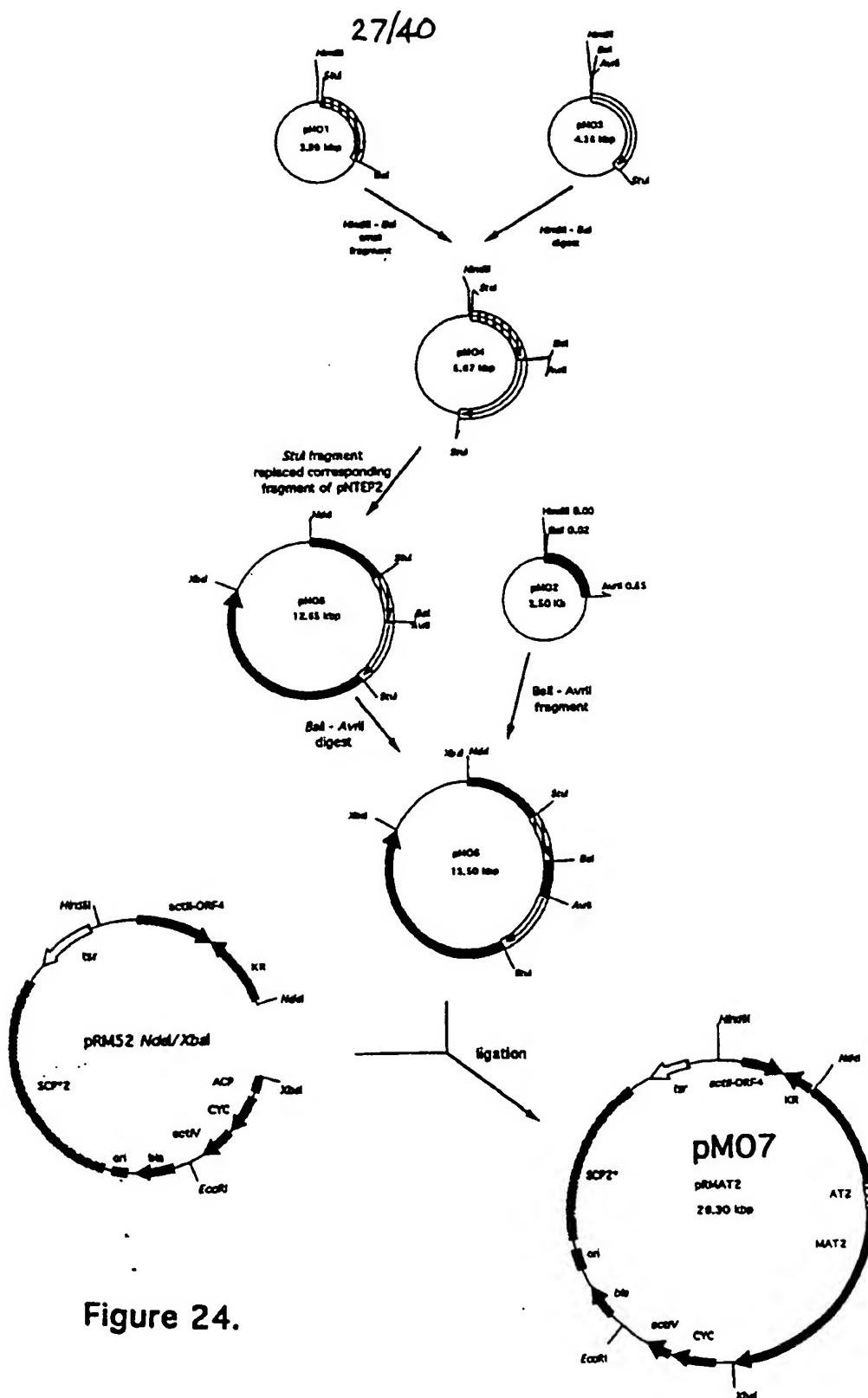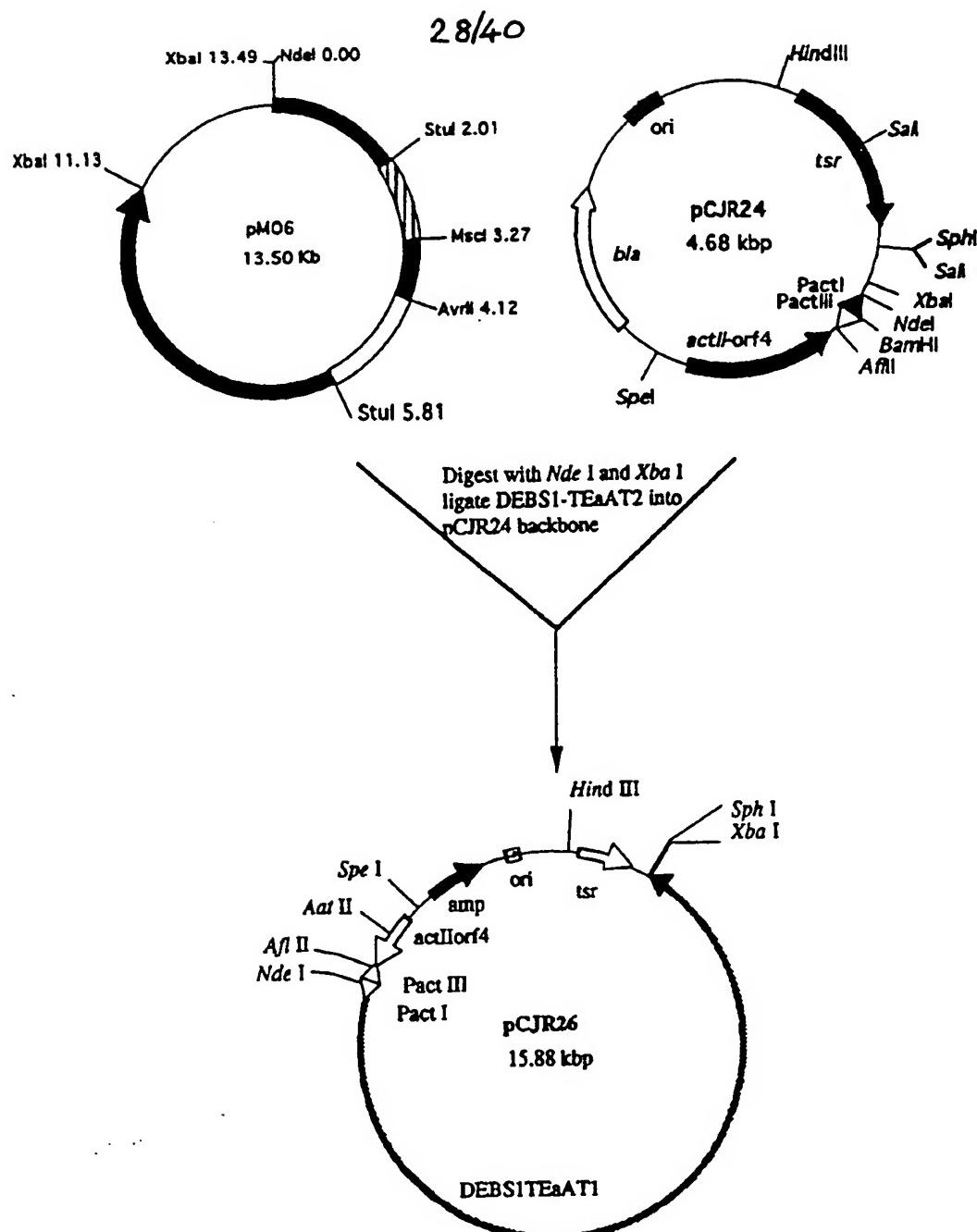
Figure
25

29/40

Figure 26

30/40

31/40

BspE I   *Bal* I   *Avr* II   *SexA* I   *Nde* I   *BspE* I   *SexA* I *Spe* I   *Xba* I

pMO32   pUC1-0

Digest with *BspE* I and *SexA* I
clone RAP AT2 into pUC1-0 backbone

Nde I   *BspE* I   *SexA* I *Spe* I   *Xba* I

pCJR43

Digest with *Nde* I and *Xba* I and clone into
pCJR24 which had previously been
digested with *Nde* I and *Xba* I

Nde I   *BspE* I   *SexA* I *Spe* I   *Xba* I

pCJR49
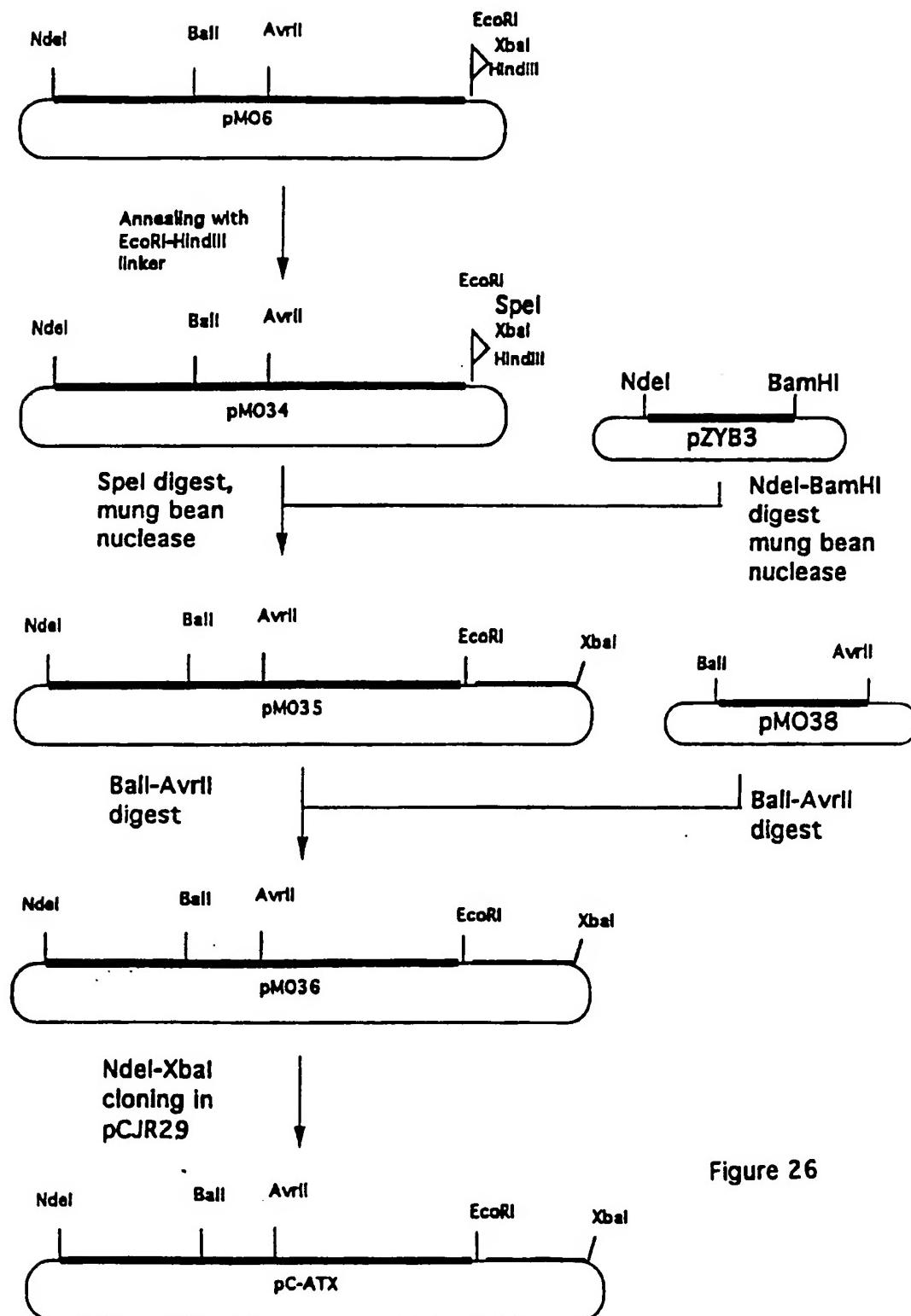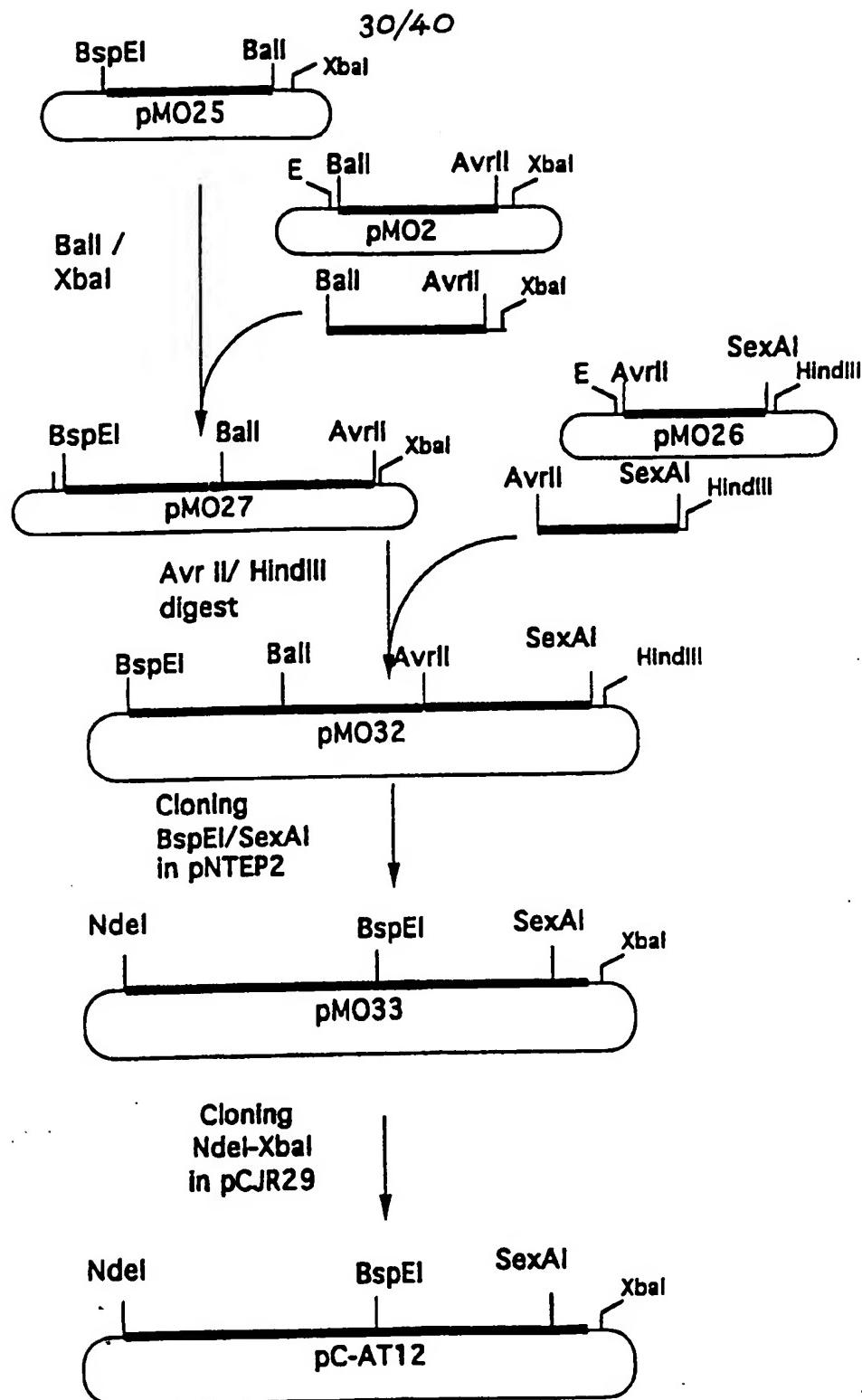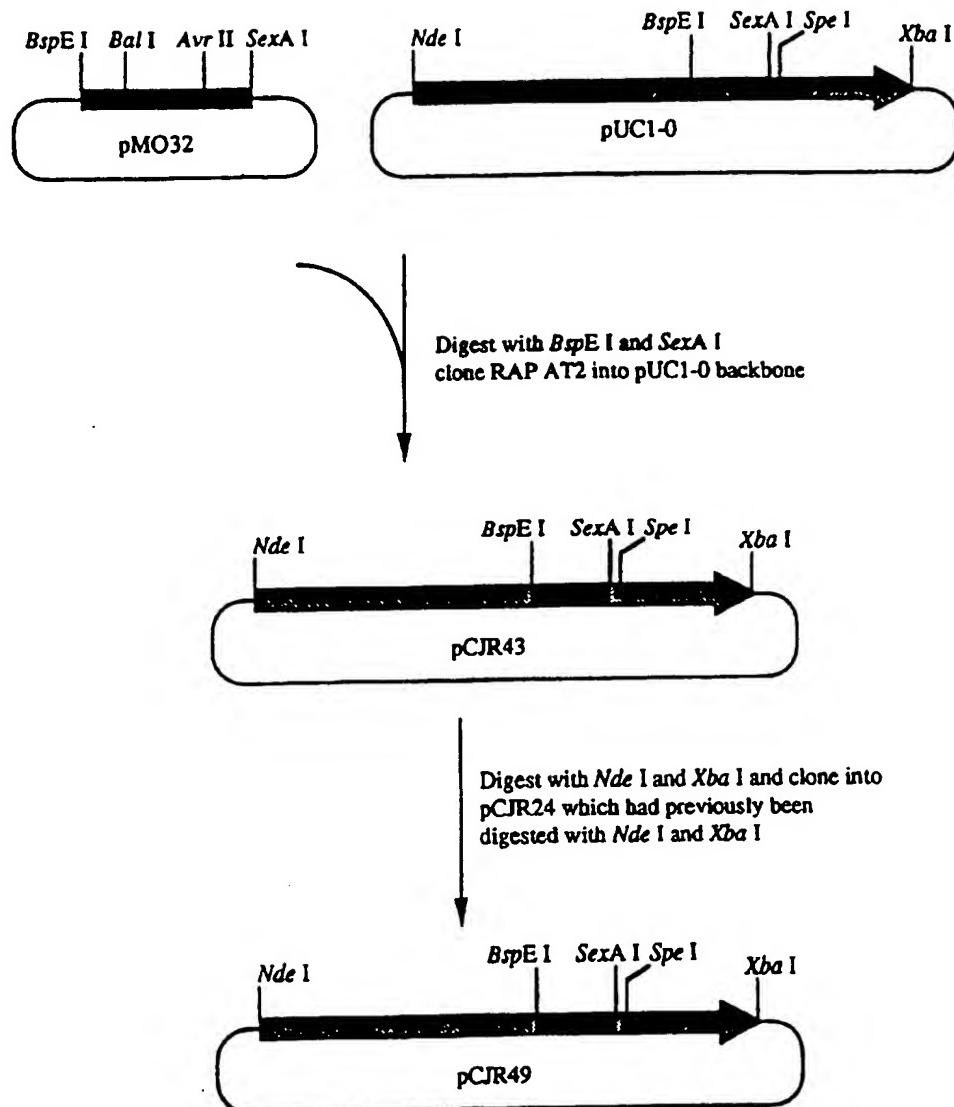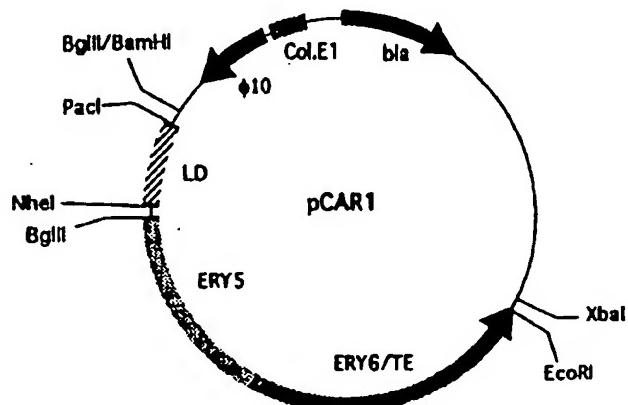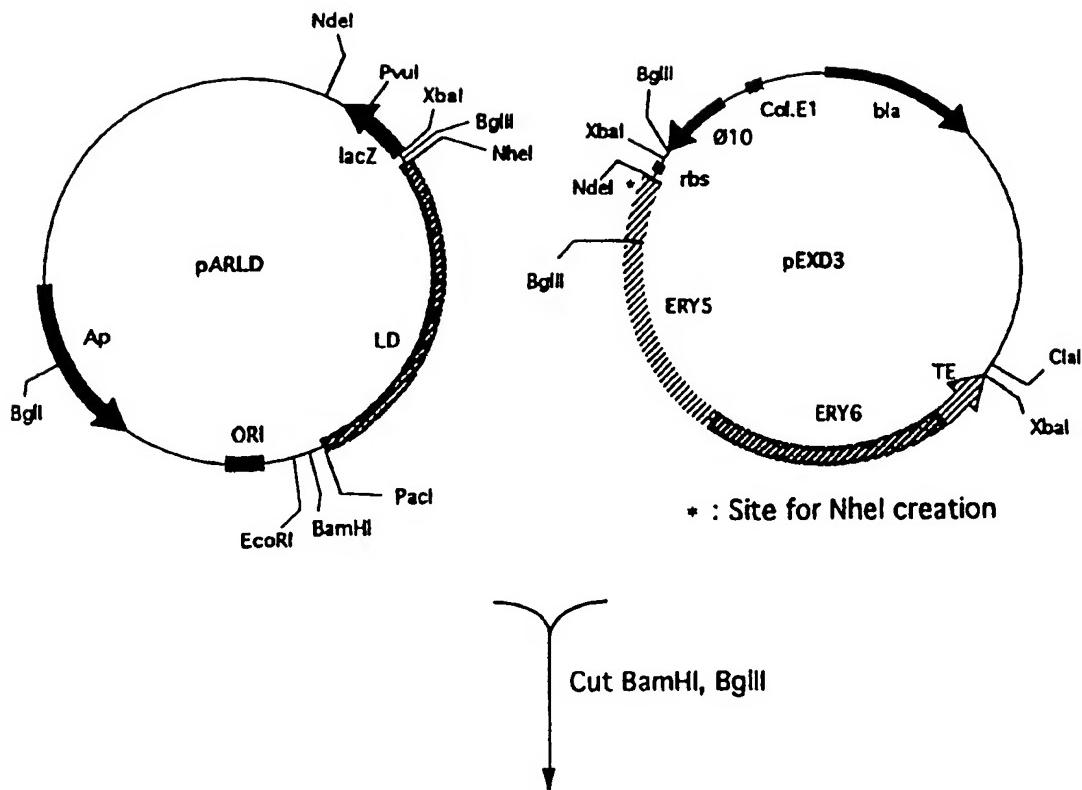
**Figure
28**

Figure 29a: Construction of pCART11
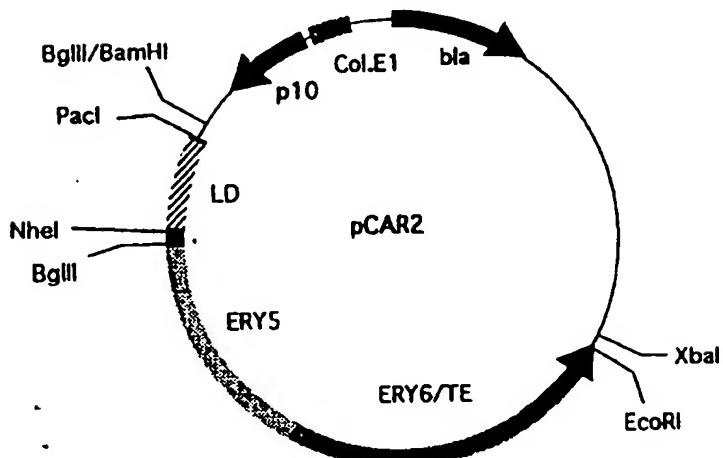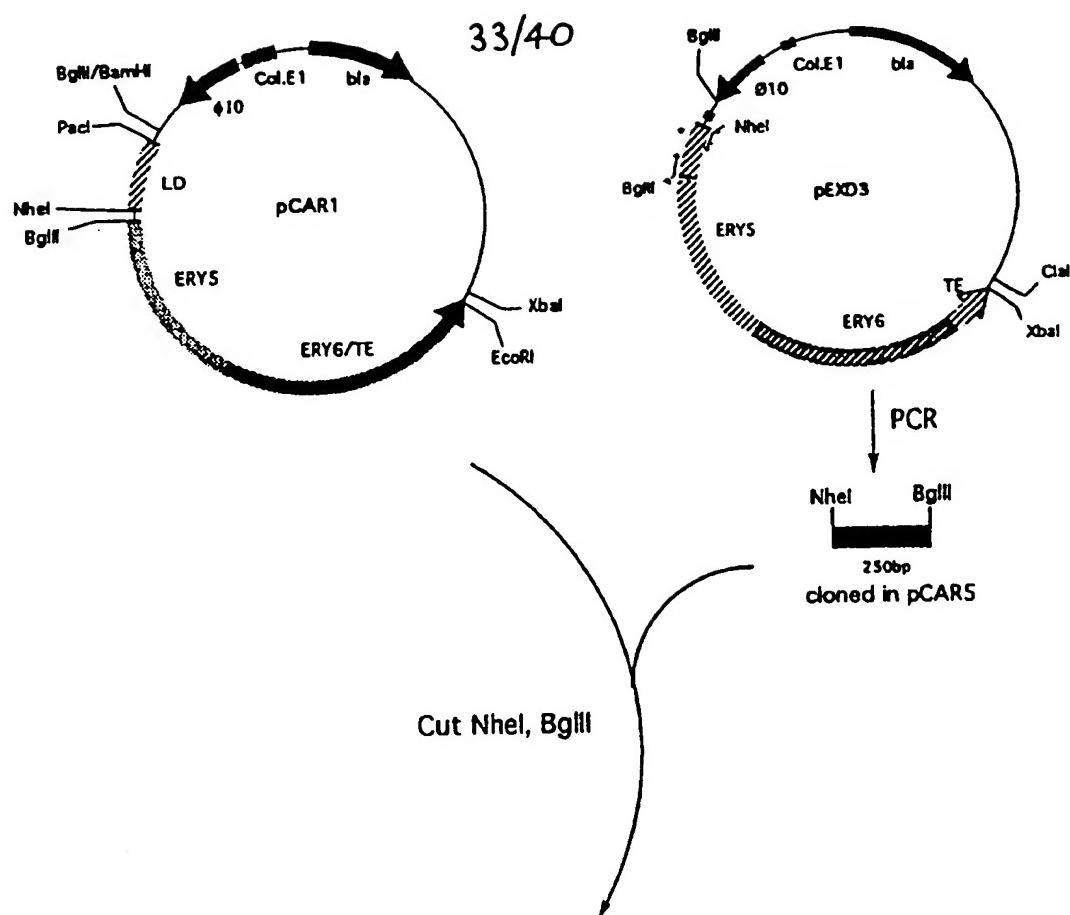
Figure 29b: Construction of pCART11
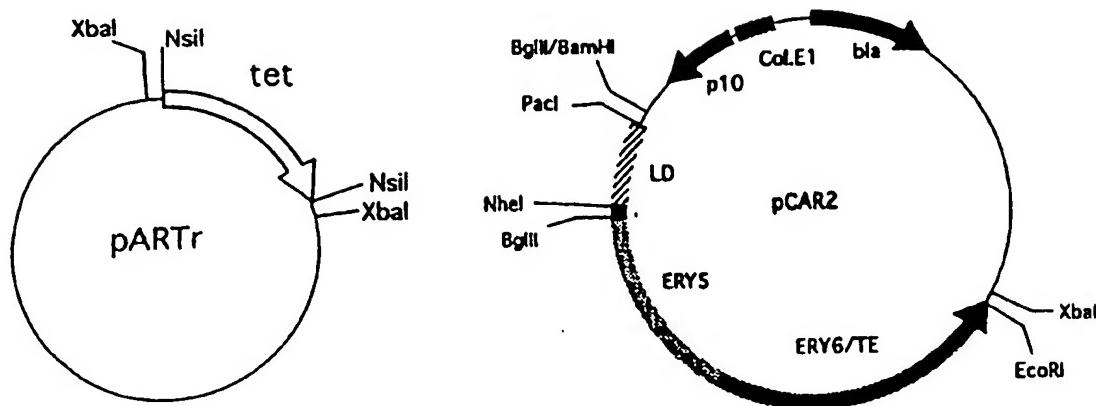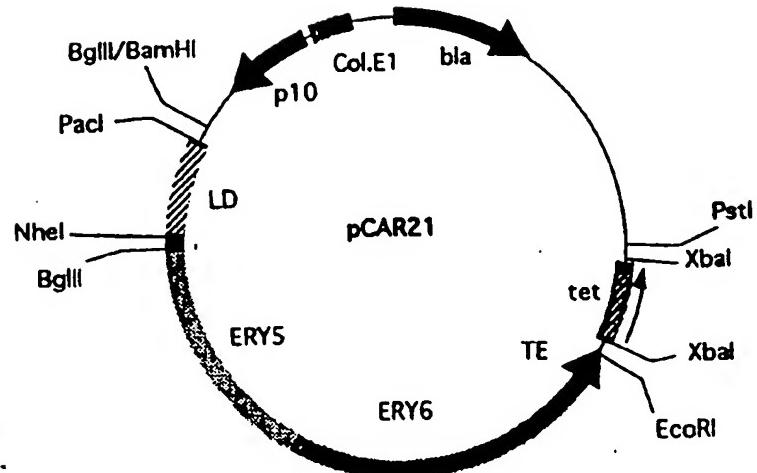
Figure 29c: Construction of pCART11

35/40
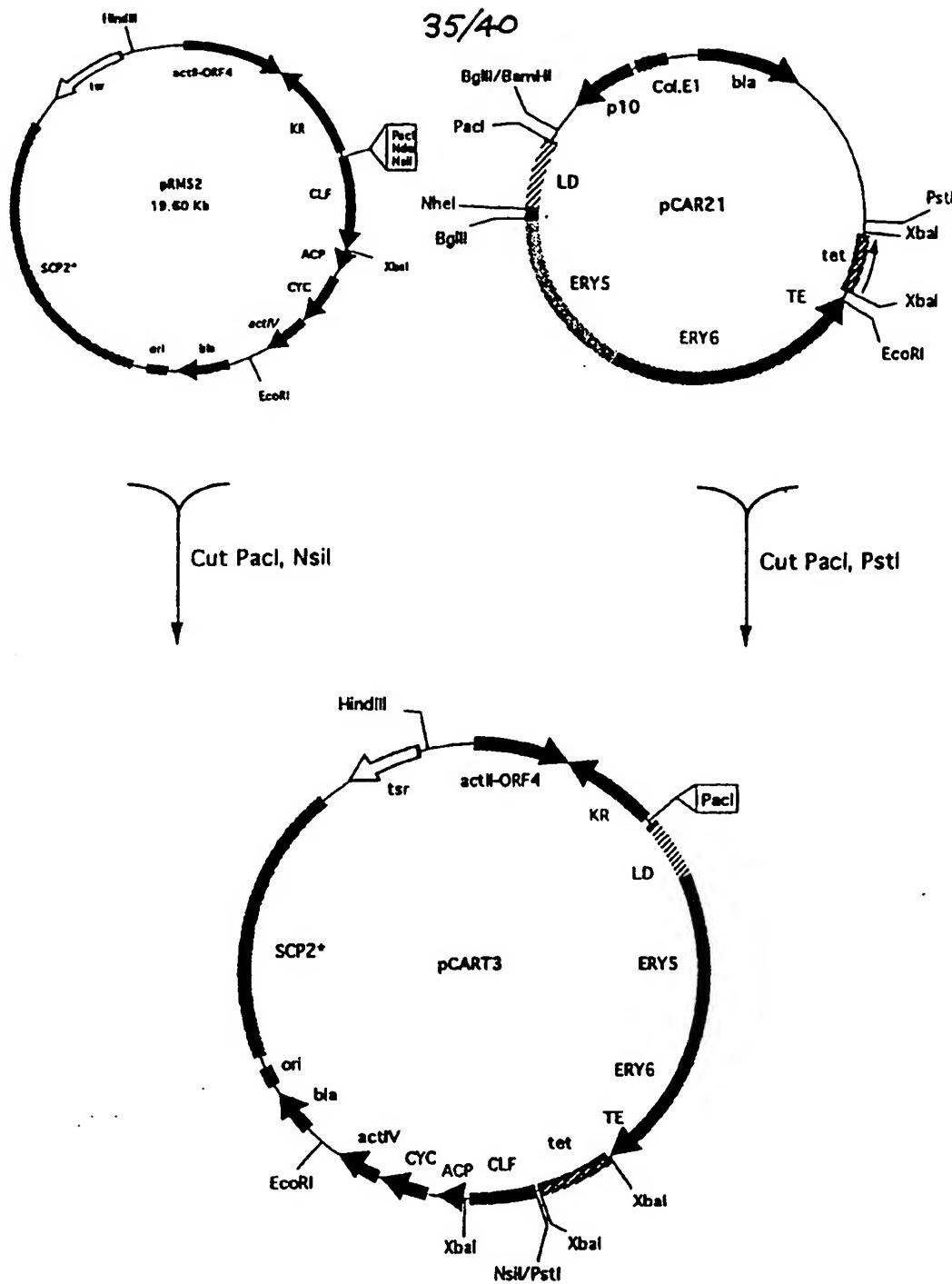
Figure 29d: Construction of pCART11

36/40
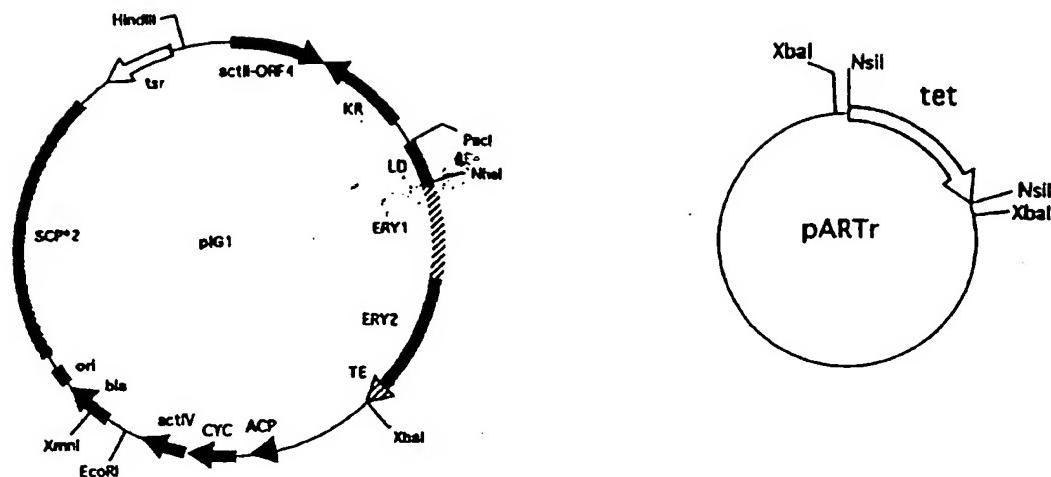
Cut Xbal
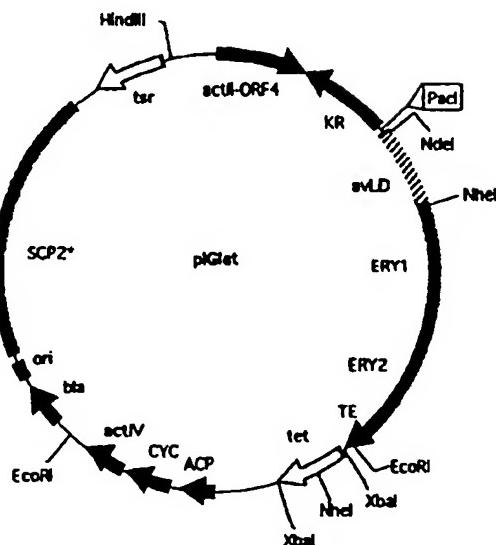
Figure 29e: Construction of pCART11

37/40

Cut NheI

Pseudo-directional cloning
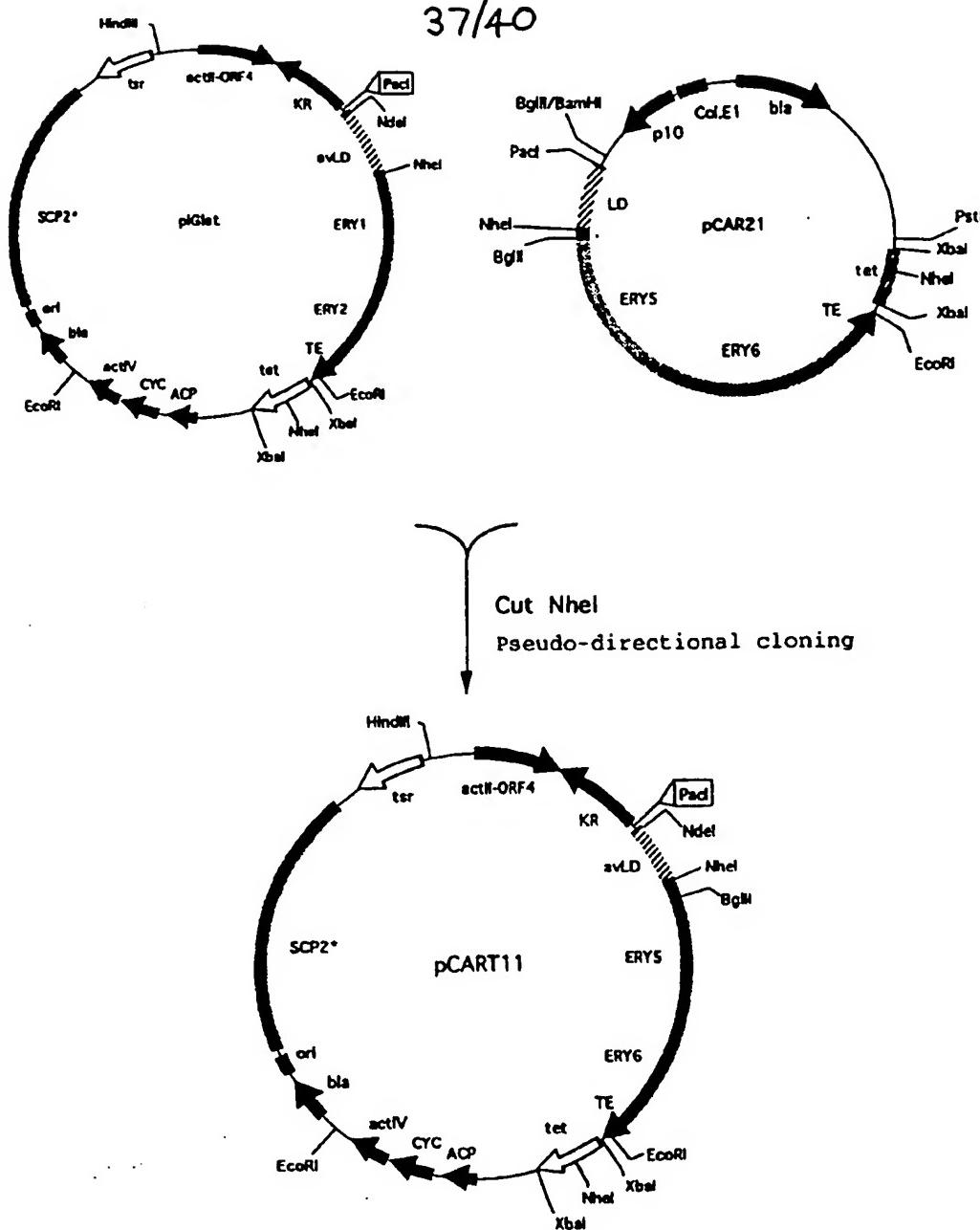
Figure 29f: Construction of pCART11

38/40

39/40

Figure 31 : Construction of plasmid pARL3

40/40

Figure 32.